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Host-pathogen crosstalk: implications in host cellular processes by intracellular pathogens

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Host-pathogen crosstalk: implications in host cellular processes by intracellular pathogens

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Editorial: Host-pathogen crosstalk: implications in host cellular processes by intracellular pathogens

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KEYWORDS

host-pathogen, intracellular, small RNA, persistence, therapeutic targets

Editorial on the Research Topic
Host-pathogen crosstalk: implications in host cellular processes by intracellular pathogens

The intricate interplay between host and pathogen is fundamental aspect of microbial pathogenesis, with far-reaching implications on human health, ecological balance, and global disease burden. In this Research Topic, we delve into the multifaceted nature of host-pathogen interactions, including the morphological and metabolic diversity of bacteria, persistence and small RNA regulatory mechanisms in intracellular pathogens like, *Mycobacterium tuberculosis*. The Research Topic also includes studies on the manipulation of host microRNAs by pathogens, the interplay between microbiota and viral infections, the genetic diversity and virulence modulation of the zoonotic parasite *Toxoplasma gondii*, and the comprehensive exploration of characteristics across various intracellular pathogens. These studies highlight the critical need for enhanced research to decipher the complexities of host-pathogen interactions and develop targeted interventions that mitigate human suffering while preserving ecosystem integrity.

Diverse pathogenic characteristics and mechanisms of a wide array of microorganisms including bacteria, viruses, protozoa, fungi and helminths, have been explored by Shukla et al.. The review highlights the intricate pathogenic mechanisms employed by these organisms, emphasizing their roles in host infections. Moreover, the authors stress upon the need for further study of both harmful and beneficial microorganisms for advancing agricultural practices and public health. The dual focus on pathogenic and beneficial microorganisms aims to address health threats while leveraging their positive aspects for sustainable development (Shukla et al.).

Soni et al. discuss the morphological and metabolic diversity of bacteria, highlighting their ecological significance and implications for human health. The authors provide insights into the structure, function, and pathogenicity of bacteria based on their classification. The intricate relationship between bacterial metabolism and host interactions underscores the complexity of microbial ecology. A multifaceted interplay of factors, primarily revolving around the host's immune system, genetic predispositions, and environmental conditions, have been found to influence the host's susceptibility to bacterial infections. The authors also address the escalating challenge of antimicrobial resistance (AMR) which poses a global threat to public health. Combatting AMR requires

a comprehensive strategy that integrates human, animal, and environmental health, emphasizing the importance of "One Health" approach (Soni et al.).

The intricate dynamics of bacterial persistence is particularly exemplified in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*, as demonstrated by the research conducted by Joshi et al.. This study investigates the multifactorial mechanisms underpinning antibiotic tolerance, biofilm formation, and macrophage survival through a transposon mutant library analysis of *M. smegmatis mc²155*. Their findings suggest that bacterial persistence is a complex phenomenon involving various metabolic pathways and stress responses. Various loci *msmeg_3233* (CydA), *msmeg_0719, bioB, msmeg_0392* and *msmeg_2263* (hybC) were identified to play critical roles in energy production, stress management, and survival strategies, highlighting their potential as targets for novel therapeutic interventions against persistent infections. The study underscores the necessity of further research on these genes and their orthologs in *M. tuberculosis* to develop effective treatments for chronic infections (Joshi et al.).

Small regulatory RNAs (sRNAs) in *Mycobacterium tuberculosis* (Mtb) form a complex regulatory network that is vital for the pathogen's adaptation, virulence, and survival within the host (Garg et al.). These sRNAs modulate gene expression at transcriptional, post-transcriptional, and translational levels, enabling Mtb to endure the hostile conditions within macrophages. The differential expression of key sRNAs, such as MTS2823 and DrrS, in response to stressors like hypoxia and nutrient limitation, underscores their critical role in Mtb's pathogenesis. This intricate interplay between sRNAs, transcriptional regulators, and mRNA targets highlights the complexity of Mtb's regulatory framework. Targeting specific sRNAs associated with virulence or antibiotic resistance may reveal new strategies for addressing drug-resistant tuberculosis and enhancing treatment outcomes. Furthermore, insights gained from investigating sRNA networks in Mtb could pave way for investigating similar regulatory mechanisms in other pathogens, leading to innovative approaches for combating infectious diseases (Garg et al.).

Latent tuberculosis infection (LTBI) possesses the potential to progress to active tuberculosis (ATBI), thereby facilitating the transmission of disease. This process is intricately influenced by *Mycobacterium tuberculosis* (Mtb) through its manipulation of host microRNAs (miRNAs). Exosomal miRNAs, particularly miR-155, miR-125b, and miR-29a, are crucial in modulating the immune response to tuberculosis (TB) by influencing macrophage and T cell differentiation. Their stability makes them valuable biomarkers for distinguishing active TB from latent infections. miRNA-based therapies show promise in enhancing immune responses and combating drug resistance. The role of exosomal miRNAs in TB underscores the potential for innovative diagnostic and therapeutic strategies, which could significantly improve patient outcomes and address the challenges posed by drug-resistant strains (Mukhtar et al.).

Recent studies investigating the role of transcriptionally active microbes (TAMs) in the serum of dengue-positive individuals using RNA sequencing by Yadav et al., revealed distinct microbial compositions linked to viral loads. High viral loads were found to be correlated with increased opportunistic microbes like *Campylobacter,* while low viral loads, associated with milder symptoms, showed more commensals such as *Lactobacillus*. Additionally, distinct lymphocyte and neutrophil counts were observed between the groups, suggesting that blood parameters and specific microbial patterns could serve as prognostic markers for disease progression. These findings highlight the importance of considering both microbiome and host factors in assessing dengue progression and developing interventions, offering a new perspective on combating this global health challenge (Yadav et al.).

Recent studies on *Toxoplasma gondii* have revealed intriguing strain-specific effects on host cells. This genetically diverse parasite, known for modulating host functions, shows varying impacts across its three main lineages. Notably, strains Me49 and NED induce host cell cycle arrest and chromosome missegregation, differing from the haplotype I (RH strain). Both strains increase binucleated cell formation, indicating cytokinesis failure, while NED uniquely alters cyclin B1 expression, indicating distinct host adaptations across haplotypes. These findings highlight complex host-parasite interactions and their potential influence on severity of toxoplasmosis. Such insights into strain-specific mechanisms may guide future research and inform targeted interventions in both human and veterinary medicine, addressing this significant public health concern (Rojas-Barón et al.).

Recent research has identified promising peptides that inhibit *Leishmania* invasion of host cells, a crucial step in leishmaniasis infection. Using phage display technology, Verga et al. identified two effective peptides that inhibit the interaction between metacyclic promastigotes (MPs) of Leishmania species and phagocytic host cells: La1, specific to *L. amazonensis*, and Li1, a dual-targeting peptide. Both have been found to reduce parasite internalization by 44% *in vitro*, while Li1 decreased visceral leishmaniasis infection in mice by 84%. These findings open new avenues for developing targeted treatments against this neglected tropical disease, potentially revolutionizing our approach to leishmaniasis management (Verga et al.).

Addison et al. recently investigated the role of geographic variations in the pathogenicity of *Photorhabdus asymbiotica* isolates. They found that, while European soil isolates lack mammalian cell survival, Australian and North American clinical strains selectively infect human immune cells, with infectivity dependent on growth temperature. A new clinical strain, *P. luminescens* was also found to infect human cells. These findings illuminate the role of geographic differences in development of virulence mechanisms, further expanding our understanding of pathogenic potential of this genus (Addison et al.).

These explorations of host-pathogen crosstalk offer critical insights into the pathogenic mechanisms influencing host cellular processes. As research continues to unravel the multifaceted relationships between hosts and pathogens, it becomes evident that a comprehensive approach—integrating ecological, genetic, and immunological perspectives—is essential for addressing public health challenges. By focusing on both harmful and beneficial microorganisms, we can develop sustainable practices that address health threats while harnessing the positive aspects of microbial diversity. This holistic understanding will be crucial for advancing therapeutic

outcomes and improving disease management strategies in the future.

Author contributions

DT: Writing – original draft, Writing – review & editing. RG: Writing – original draft, Writing – review & editing.

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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Dual RNA-Seq reveals transcriptionally active microbes (TAMs) dynamics in the serum of dengue patients associated with disease severity

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Introduction: Dengue virus (DENV) is a flavivirus that has emerged as a global health threat, characterized by either asymptomatic or mild self-limiting febrile illness, but a subset of DENV outbreaks have been associated with severe disease. Studies have looked into the host immune response and dengue viral load during infection. However, it remains unknown how the active microbial isolates modulate the dengue viral infection. In this study, we demonstrate the significance of in-depth analysis of microbiota composition in the serum samples of dengue-infected patients.

Materials and methods: RNA was extracted from the serum samples collected from 24 dengue positive patients. The human mapped reads generated through RNA-Sequencing (RNA-Seq) were removed, while the unmapped (non-human) reads were employed for microbial taxonomic classification using Kraken2 and Bracken2. Further, we assessed the initial blood parameters analyzing the complete blood count (CBC) profile of the patients.

Results: Findings revealed differential abundance of commensals and pathogenic microbes in the early febrile period of hospitalized dengue patients, segregated into, High Viral Reads (HVR) and Low Viral Reads (LVR). The Campylobacter genus was abundant in the HVR whereas Lactobacillus dominated the LVR patients. At species level, the microbiota of HVR exhibited higher abundance of unique potential opportunistic microbes, compared to the commensal microbes' enrichment in the LVR patients'. We hypothesize that the DENV might alter the microbiota composition as observed by the increase in preponderance of opportunistic pathogens and an absence of commensals in the HVR. The presence of commensals in the LVR might explain, i) overall lower dengue viral reads compared to the HVR, and ii) shift in lymphocytes (high) and neutrophils (low) counts; resulting in a comparatively milder clinical manifestation in this group. Our findings may help in understanding the co-infection aspect that will be important to develop dengue therapeutics and vaccines.

Discussion: This study highlights the potential of the unexplored roles of the TAMs in modulating the dengue disease severity using the metatranscriptomic sequencing. This study serves to enhance our understanding of the distinctive

microbial and hematologic signatures in the early infection stage that differentiate patients with high viral reads patients from those with low dengue viral reads.

KEYWORDS

dengue infection, high and low dengue viral reads, clinical parameters, dual RNA-Seq, transcriptionally active microbes, disease severity

Importance of the study

Each year, India records more than a hundred thousand dengue cases resulting in immense public health burden. Here, we employed the RNA-Seq approach to explore the functional dynamics of transcriptionally active microbes (TAMs) in the serum of dengue positive individuals. We discovered that the patients who had high dengue viral load had an elevated number of opportunistic microbial species and were devoid of commensals. Contrarily, the balanced presence of pathogenic/opportunistic and commensals plausibly explains the milder clinical manifestations of LVR. In the early febrile period of Dengue, we observed a distinct demarcation of Lymphocyte and Neutrophil counts in blood parameter profiles among dengue patients between the two groups. These findings suggest that the use of blood parameters as prognostic markers, along with the identification of specific microbial patterns associated with disease severity can be used to predict disease progression.

Introduction

Dengue virus (DENV), comprising four distinct serotypes (DENV 1–4), causes dengue infection which is one of the most important yet highly neglected tropical diseases in the world. The incidence of dengue has alarmingly increased over the past few decades, rising from 0.5 million cases in 2000 to 5.2 million in 2019 (Gutierrez-Barbosa et al., 2020). The diverse range of symptoms observed underscores the need to comprehend the underlying factor/s contributing to the severity of clinical manifestations. Notably, clinical parameters, including laboratory tests such as complete blood count (CBC), serological tests, and blood culture, along with viral antigen positivity, can be utilized to confirm the diagnosis as well as assess dengue disease severity. While an abnormal platelet count and function have been recognized as hallmarks of dengue infection, other parameters, like low lymphocyte percentage, are associated with variable disease severity (Low et al., 2011; Michels et al., 2014; Mukker and Kiran, 2018; Yadav et al., 2023).

Considering the diversity in viral genotype and clinical parameters among dengue patients, most of the studies have explored viral and host factors to define dengue infection and its impact on clinical phenotypes, seeking biomarkers of severity. Coincidentally, few studies have also reported an increased prevalence of bacterial infections, followed by dengue viral infections (JCDR, 2018; Lee J. C. et al., 2022; Yadav and Pandey, 2022). The scanty information, through case studies, gives an insight into the importance of co-infection along with the primary dengue infection (Miyata et al., 2015; de Filippis et al., 2016). COVID-19 pandemic has also highlighted the functional role of co-presence of *transcriptionally active microbes (TAMs)* as a major disease modulator (Devi et al., 2022, 2023a,b).

This pilot study focuses on the impact of microbial co-presence during dengue infection. The serum from dengue positive patients ($n = 24$) who reported to MAX Hospital, Delhi, was collected and using metatranscriptomic sequencing, microbial co-infections were comprehensively evaluated. The median age of these patients was 21.5 years with the minimum and maximum age of 3 and 68 years, respectively. Also, 66.7% ($n = 16$) of the patients were male as compared to 33.3% ($n = 8$) females.

Materials and methods

Patient sample collection

The study was carried out at CSIR-Institute of Genomics and Integrative Biology (CSIR-IGIB) in collaboration with MAX Healthcare Hospital, Delhi, India. Dengue positive patients ($n = 24$) who reported to MAX Healthcare Hospital, Delhi, India, were recruited into this study. After collecting blood from these patients, serum was separated from the blood and tested for dengue NS1Ag. All samples had a ratio of >1.00 for the Dengue NS1Ag Test. Dengue viral detection was done from the serum sample collected by the paramedical staff at the hospital on the day of reporting. Bio-Rad Platelia Dengue NS1 Ag test was used for detecting DENV using the Dengue NS1 Antigen Test (ELISA).

Serum RNA isolation, library preparation and sequencing

Viral RNA was isolated from the serum using QIAamp RNA Blood Mini Kit (cat. No. 52304) which was used for RNA-Seq. The library preparation protocol for RNA-Seq has been previously published from our lab (Yadav et al., 2023). Briefly, a total of 250 ng of RNA were utilized for library preparation using the Illumina TruSeq Stranded Total RNA Library Prep Gold (Illumina, Cat. No. 20020598). The final library was quantified using a Qubit double-stranded DNA (dsDNA) high-sensitivity (HS) assay kit (Thermo Fisher Scientific; catalog no. Q32854). The quality of cDNA libraries was checked using the Agilent 2100 Bioanalyzer. A final loading concentration of 650 pM was used for sequencing using NextSeq 2000, with paired-end 2 × 151 reads.

RNA pre-processing and bioinformatic analysis

Base call files from sequencer were converted into FASTQ format using bcltofastq. FastQC and Trimmomatic-0.36 were used to check the quality of the reads and remove adapters as well as low quality

reads (Bolger et al., 2014), respectively. The high quality trimmed reads were aligned to the reference human transcript using HISAT2 to remove human host RNA reads. For the human unaligned (microbial) reads, Kraken2 (Wood et al., 2019) and Bracken2 (Lu et al., 2017) were used to infer microbial communities. Bracken2 (Bayesian Reestimation of Abundance with KrakEN) was performed to identify the Dengue reads across the samples, using the non-human reads from the RNA-seq data. The Kraken2 database was used to create a Bracken-compatible database using the brackenbuild function, and the Kraken2 report files for each sample were run against the Bracken database using the bracken function for the phylum, genus and the species level information. Taxonomic Diversity Analysis through Alpha and Beta diversity were performed using the phyloseq (v1.27.2) and vegan (v2.5–4) packages in R (v3.4.3). To further analyze the beta diversity, we utilized Bray-Curtis distance matrices and performed principal coordinate analysis (PCoA), where PC1 accounted for 24.31% of the variance and PC2 for 10.22%.

Statistical analysis

Statistical analysis for clinical parameters were done through Mann–Whitney test using Graph pad prism. For Beta diversity analysis, PERMANOVA/adonis2 test in R (using the Vegan (v2.5–4) package) was calculated to determine the statistical significance. To remove count data bias, we employed MetagenomeSeq's Cumulative Sum Scale (CSS) algorithm for data normalization. STAMP software with Welch's test, effect sizes, and 95% confidence intervals was used to screen statistical significance for common species across HVR and LVR patients.

Dengue serotype classification

Trimmed Fastqs were mapped to DENV Serotypes 1–4, using HISAT2. The final serotype of a particular sample is determined by the serotypes with the highest percentages in that sample. BAM was converted into consensus FASTA using bcftools.

Availability of dataset

The datasets generated during the current study are available in the NCBI SRA under the access number PRJNA955953.

Results

Change in hematological characteristics (lymphocytes and neutrophils) as predictive markers for dengue disease outcome in early febrile period

RNA-Seq generated 12,964,528 raw sequencing reads, which were categorized into two main categories: Human and Non-human reads. The non-human reads were analyzed using Kraken2 followed by the Bracken2, a k-mer based taxonomic classification tool, for identification of microbial presence using Bayesian re-estimation of abundance. In total, 24 samples generated an average of ~2,775,261 microbial reads/sample. Our analysis revealed an intriguing unequal distribution of dengue virus reads, which prompted us to divide them into two distinct groups: High viral reads (HVR) and Low viral reads (LVR) with 12 samples each (Supplementary file S1). For HVR and LVR patients, the median ages were 25 and 36, respectively.

Further, we identified DENV Serotypes from the non-human RNA-seq reads in 16/24 samples, of which 12 samples with high DENV reads were serotype positive, while 4 samples from the LVR were positive for serotype identification. Simultaneously, we conducted a comprehensive analysis of CBC parameters in patients corresponding to the two groups (Figure 1A). Analysis allowed us to explore how these parameters were influenced by dengue infection. In terms of platelet and total leukocyte count, which are considered critical hematological features for predicting dengue disease outcomes, no significant differences have been found. Can lymphocytes and neutrophils be used as potential predictive markers for disease progression, since we observed a significant difference for these two parameters? In contrast to the LVR group, lymphocytes and neutrophils in the HVR patients displayed low and high values, respectively (Figure 1A). The shift in lymphocytes count has previously been associated with dengue infection, studies have highlighted that the patients with early disease had significantly lower lymphocyte counts (Sigera et al., 2019). The evidence, however, is still up for debate as to how neutrophils contribute to the disease progression. While a few studies reported early-phase neutropenia in dengue patients, a higher neutrophil percentage predominantly in the first 5 days of the fever has been revealed by a retrospective study (Chaloemwong et al., 2018). The study additionally identified a negative correlation between decreased neutrophil and increased lymphocytes count, emphasizing the neutrophil to lymphocyte ratio as > 1 on the first 5 days. Further research is required to understand the functional implications of neutrophil levels and dengue disease outcomes.

Overview of TAMs diversity and phyla and genera level microbial community composition

To gain insights into the microbial genomes alongside the dengue genome, we focused on analyzing the non-human reads. Using taxonomic classification analysis, we mapped 4,859 TAMs from the bracken database.

The analysis revealed the presence of bacterial, viral, and archaea bacterial genomes. Bacteria was the predominant microbial community (77.1%), followed by viruses (18.7%) and archaea (4.25%) (Figure 1B). A significant distribution in the viral and bacterial populations was observed across the two groups, but not for archaea (Supplementary Figure S1A). We further proceeded to compare the alpha and beta diversity metrics of patient microbiomes in the HVR and LVR. Incidentally, we did not observe significant differences in within-sample (alpha) and between-sample (beta) diversities of the microbiomes between HVR and LVR patients. However, there was a notable difference in the Chao1 index, statistically significant ($p = 0.04$, Kruskal-Wallis test) (Figure 1C) (Supplementary Figure S1B). Beta diversity analysis also showed non-significant clustering patterns between the two groups when visualized in a PCoA plot.

A

Parameters	*HVR*[$] *(n=12)*	*LVR*[$] *(n=12)*	*p value*
Age (IQR)	25(39-65)	36(24-56)	0.088
Gender[%]	5/7	3/9	0.386[b]
Viral serotype[#]	3/7/0/2/0	1/2/1/0/8	-
NS1 Antigen ratio in serum	3.4	3.5	0.841
Lymphocytes	13.2	35	**0.001***
Neutrophils	77.4	56.7	**0.002***
RBC count	4.4	4.9	0.347
MCV	89	85.4	0.2
MCH	29.5	28.15	0.2
MCHC	32.85	32.25	0.7
MPV	8.65	10.1	0.1
RDW	14.15	13.75	0.7
Monocytes	8.15	9.25	0.7
Basophils	0.35	0.4	0.6
Hb	12.6	13.3	0.8
Platelet Count	167.5	185	0.9
RBC Count	4.385	4.975	0.3
Total Leucocyte Count (TLC)	4.45	4.05	0.4
Eosinophils	0.2	0.9	0.3
Packed Cell, Volume	39.9	39.15	0.9

$median
#DENV-1/DENV-2/DENV-3/DENV-4/unknown
%Female/Male
Statistical significance calculated using Mann Whitney U test and [b]Chi2 test.
Values of significance are highlighted in bold.

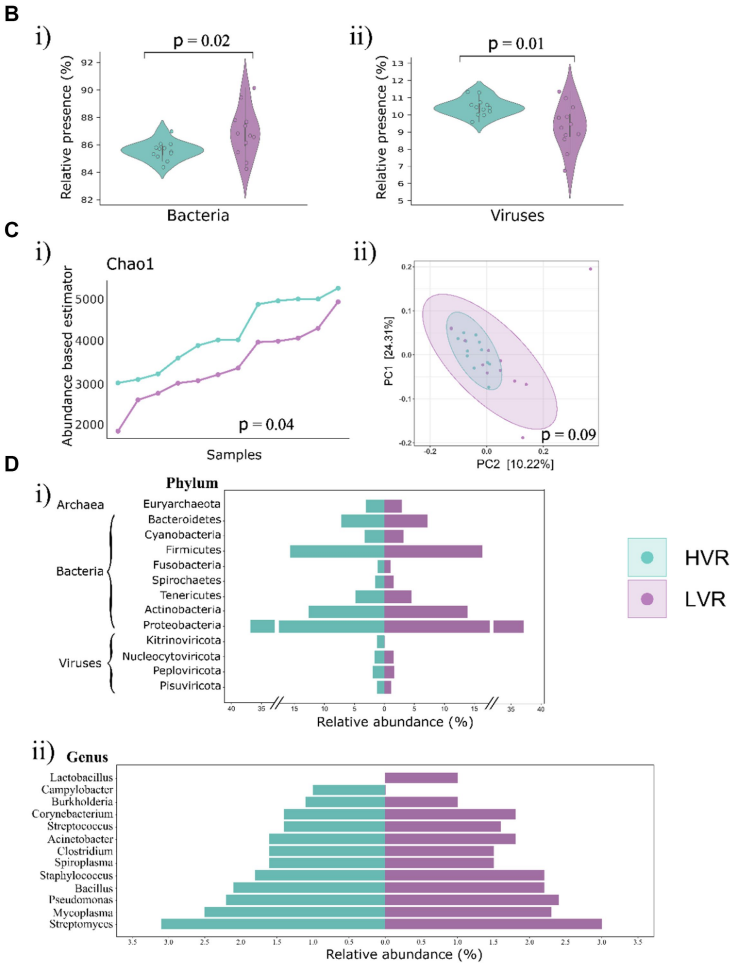

FIGURE 1
Dengue patients' clinical data overview, microbial abundance and diversity. **(A)** Key demographic features, viral serotype and clinical characteristics (CBC) of the dengue patients. **(B)** Violin plots showing the relative abundance of **(i)** bacteria, and **(ii)** viruses. **(C)** Visualization of alpha and beta diversity, **(i)** Chao-1 (Abundance-based estimator) alpha diversity index with *p*-values calculated by the Kruskal Wallis test, **(ii)** Beta diversity wherein Principal Coordinate Analysis (PCoA) shows the differential composition of the microbes across HVR and LVR. **(D)** Illustration of percent relative abundance at **(i)** phyla and **(ii)** genera level identified in the two groups.

For in-depth analysis of the microbial composition, we focused on understanding the relative abundance of phyla and genera in both the groups, which showed striking similarities (Supplementary Table S2). Notably, we observed that *Proteobacteria* (36.8% in HVR vs. 37.1% in LVR), *Actinobacteria* (12.6% in HVR vs. 13.8% in LVR), and *Firmicutes* (15.7% in HVR vs. 16.3% in LVR) were prevalent and equally distributed phyla in both HVR and LVR. In terms of viral phyla, *Nucleocytoviricota*, *Peploviricota*, and *Pisuviricota* exhibited no significant differences between the HVR and LVR, except for the phylum *Kitrinoviricota*, which dengue virus belongs to (Figure 1Di).

Further narrowing down the analysis for genera, *Lactobacillus* showed abundance in the LVR (1% LVR vs. <1% HVR), while *Campylobacter* exhibited abundance in the HVR (1% HVR vs. <1% LVR). The *Campylobacter* genus are a diverse group of bacteria, mostly regarded as important human pathogens (Facciolà et al., 2017). Contrariwise, the *Lactobacillus* comprises the beneficial species that are generally a major part of human microbiota, including the digestive and female genital system (Fijan, 2014). However, we also observed shared characteristics in the abundance of certain genera among both dengue sub-groups (Figure 1Dii). The genera characterized by higher relative abundance differences in both the groups are highlighted in Table 1. Although not so stark, we uncovered significant differences in the prevalence of commensal and opportunistic microbial taxa at the genera-level between HVR and LVR groups.

Differential presence of commensals and opportunistic active microbial species reveals their putative role in disease trajectory

The presence of functionally distinct genera in the two groups intrigued us to delve deeper at the species level. To ensure stringency of our findings, the species with a relative cumulative abundance of <0.05% and presence in <50% of the samples were excluded, resulting in a dataset of 607 species.

From common TAMs...

We specifically examined the species that were shared between the HVR and LVR. Of the total species identified, we found 470 common species between the groups. Using the STAMP tool for significance, 16 species showed notable differences. Heatmap depicts the varying abundance of these species in the HVR and LVR (Figure 2A). Interestingly, two species, *Cutibacterium granulosum* and *Staphylococcus capitis*, were found to be abundant in the LVR, while the remaining 14 species, including *Dengue virus*, showed abundance in the HVR. It is worth mentioning that certain species identified in our study, such as *Fusobacterium varium* and *Staphylococcus capitis*, have been reported as pathogenic or opportunistic bacteria associated with human infections (Mayslich et al., 2021; Chong et al., 2022; Lee S. J. et al., 2022). Although *Campylobacter sputorum* itself is not directly linked to disease, the *Campylobacter* genus has been associated with clinical conditions such as watery stools with fever, abdominal pain, vomiting, and dehydration, all of which are symptoms commonly observed in dengue infection (Mazaheri et al., 2016).

TABLE 1 Distribution of genus present at >1% relative abundance in two groups.

Genus	HVR (%)	LVR (%)
Streptomyces	3.1	3
Mycoplasma	2.5	2.3
Pseudomonas	2.2	2.4
Bacillus	2.1	2.2
Staphylococcus	1.8	2.2
Clostridium	1.6	1.5
Acinetobacter	1.6	1.8
Spiroplasma	1.6	1.5
Streptococcus	1.4	1.6
Corynebacterium	1.4	1.8
Burkholderia	1.1	1

...to unique TAMs

The identification of potential TAMs as biomarkers that could explain the association between HVR and an increased disease progression needs to be evaluated. We investigated the unique microbial species between two groups. In the HVR, we identified 68 unique species, while the LVR had 69 unique species. Interestingly, we observed that all 69 species in the LVR belonged to bacteria, whereas in the HVR, there were 56 bacteria, along with 9 viruses and 3 archaea. Findings revealed that the HVR was predominantly characterized by the presence of opportunistic or potential pathogenic bacteria, namely *Capnocytophaga gingivalis*, *Burkholderia stabilis*, *Bacillus cytotoxicus*, and few emerging pathogens that are commonly reported in immunocompromised patients, including *Methylobacterium radiotolerans*, *Listeria ivanovii*, *Borrelia parkeri*, *Helicobacter cinaedi*, *Corynebacterium falsenii*, and *Exiguobacterium* sp. *AT1b* (Ehrmann et al., 2016; Cordovana et al., 2019; Cairo et al.,

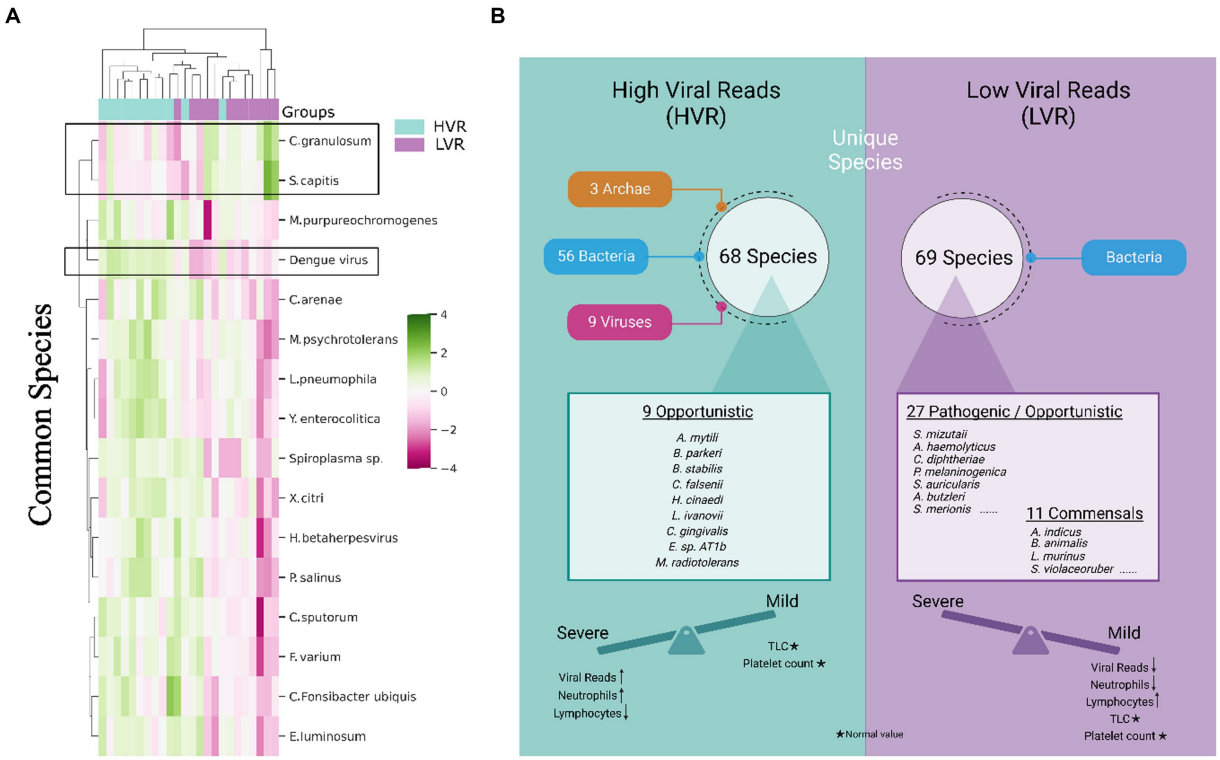

FIGURE 2
Transcriptionally active microbial species spectrum across the HVR and LVR Dengue patients. **(A)** Heatmap represents the differential abundance of 16 significant common bacterial species across the 2 groups. **(B)** Functional illustration highlighting the unique microbial species present in the HVR and LVR and their role toward shifting the disease trajectory to mild or severe along with the clinical information.

2022). Surprisingly, the presence of commensals, such as *Bifidobacterium animalis*, *Lactobacillus crispatus*, *Lactobacillus murinus*, *Neisseria elongata*, *Neisseria subflava*, and *Streptomyces violaceoruber* was found within the LVR whereas such commensal species were completely absent in the HVR (Jungersen et al., 2014; Kim et al., 2019; Pusparajah et al., 2021) (Figure 2B). Notably, species such as *Bifidiobacter animalis* and *Lactobacillus murinus* in the LVR function as probiotics, known to inhibit pathogens (Jungersen et al., 2014; Huang et al., 2016).

The LVR exhibits a noteworthy balance between opportunistic and commensal TAMs, which, coupled with decreased dengue viral reads, potentially plays a role in mitigating the severity of the disease. Additionally, we noticed an increased presence of various rare species, including marine and plant species, in the HVR, which might suggest a correlation with patients' dietary habits. Overall, our functional characterization of transcriptionally active opportunistic/pathogenic and commensal microbial species helps us understand the underlying reasons for the clinical differences between HVR and LVR patients in terms of disease severity.

Discussion

With the lessons learnt from the COVID-19 pandemic, it is extremely crucial to understand the role of co-presence/co-infection of microbes in addition to primary infection agent, with disease severity. In the present pilot study, we investigated 24 dengue patients with high and low dengue viral load. To the best of our knowledge, this is the first study which reveals the dynamics of TAMs in the serum RNA of dengue patients, captured through host RNA-seq. Our modified meta-transcriptomic strategy identified higher abundance of rare and opportunistic bacterial species in HVR.

Of note, studies in other viral infectious diseases, influenza and COVID-19, have revealed functional impact of microbes in modulating disease trajectory. Few studies have explored the microbial community structure in *Aedes* mosquito, a vector for dengue virus (Rodpai et al., 2023). Till now, there is no published metatranscriptome study on dengue co-infection within the human host, except for a few studies highlighting concurrent bacterial co-infection in adult patients with dengue fever (Lee et al., 2005; Trunfio et al., 2017).

Our results revealed a remarkable difference in the unique microbial community between HVR and LVR. The HVR harbors a higher abundance of rare species. The presence of transcriptionally active isolates of opportunistic microbial species might be playing a modulatory role in disease severity, as exemplified by the presence of *Burkholderia stabilis*, *Capnocytophaga gingivalis*, and *Arcobacter mytili* in the HVR, where *B. stabilis* is an opportunistic pathogen that cause nosocomial bloodstream infections (Otağ et al., 2005). Also, *C. gingivalis* has been described as a multidrug-resistant bacteria, associated with acute exacerbation of chronic obstructive pulmonary disease (COPD) in an immunocompetent patient (Ehrmann et al., 2016). The LVR, on the other hand, exhibited a balanced presence of both pathogenic and commensal species, of which commensals were completely absent in HVR. Along with other commensals from intestinal flora (*Streptomyces violaceoruber, Bifidobacterium animalis*), vagina (*Gardnerella vaginalis*), skin (*Auricoccus indicus*), and oral cavity (*Propionibacterium* sp. *oral taxon 193*), *Lactobacillus*, which are probiotics, at both the genus and species level, were the unique species of LVR. This may aid the host to fight off the virus thereby ameliorating severe disease conditions.

Conclusion

In this study, the serum samples were predominantly sourced from 24 dengue-positive patients from a single hospital within a specific region to investigate microbial patterns during dengue infection. It's important to acknowledge that this approach has certain limitations. While this choice reduces variability within the samples, it also limits the broader applicability of our findings. These findings will be strengthened with future studies to examine the functional role of co-presence of TAMs in a larger and diverse dengue patient cohort, as well as in the blood isolated RNA, where the DENV replication occurs. Additionally, a longitudinal sampling approach of dengue patients would have provided valuable insights into the dynamic changes in active microbial populations throughout the disease trajectory. Longitudinal sampling of the dengue patients may further enhance the scope to understand the dynamics of active microbial populations during the disease trajectory. Despite that, the present study, which might point to the transition of disease trajectory (mild or severe) by the co-presence of various microbial communities along with the primary dengue virus and their distribution across the two groups, provided crucial insight into this unexplored yet important role of TAMs in dengue viral infection.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving humans were approved by the Institutional Ethics Committee of both CSIR-Institute of Genomics and Integrative Biology, and Max Super Specialty Hospital, under the approval number CSIR-IGIB/IHEC/2020-21/01. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

RP: Conceptualization, Funding acquisition, Resources, Supervision, Visualization, Writing – review & editing. AY: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. PK: Formal analysis, Methodology, Visualization, Writing – original draft. PD: Data curation, Investigation, Supervision, Writing – original draft. JA: Resources, Writing – review & editing. SB: Resources, Writing – review & editing. BT: Resources, 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1307859/full#supplementary-material

SUPPLEMENTARY FILE S1
Kraken output file showing a diverse group of bacteria and viruses for all the 24 samples, with 12 samples having high dengue virus reads and the rest showing lower dengue viral reads based on RNA-seq.

SUPPLEMENTARY FIGURE S1
(a) Relative abundance of archaeal reads in our cohort **(b)** Alpha diversity indices showing bacterial species richness (Shannon) and evenness (Simpson) across the patients in HVR and LVR.

SUPPLEMENTARY TABLE S2
Taxonomic communities at the level of phylum and genus, identified through Bracken in HVR and LVR cohort.

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Identification of genes associated with persistence in *Mycobacterium smegmatis*

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The prevalence of bacterial persisters is related to their phenotypic diversity and is responsible for the relapse of chronic infections. Tolerance to antibiotic therapy is the hallmark of bacterial persistence. In this study, we have screened a transposon library of *Mycobacterium smegmatis* mc^{2}155 strain using antibiotic tolerance, survival in mouse macrophages, and biofilm-forming ability of the mutants. Out of 10 thousand clones screened, we selected ten mutants defective in all the three phenotypes. Six mutants showed significantly lower persister abundance under different stress conditions. Insertions in three genes belonging to the pathways of oxidative phosphorylation *msmeg_3233* (*cydA*), biotin metabolism *msmeg_3194* (*bioB*), and oxidative metabolism *msmeg_0719*, a flavoprotein monooxygenase, significantly reduced the number of live cells, suggesting their role in pathways promoting long-term survival. Another group that displayed a moderate reduction in CFU included a glycosyltransferase, *msmeg_0392*, a hydrogenase subunit, *msmeg_2263* (*hybC*), and a DNA binding protein, *msmeg_2211*. The study has revealed potential candidates likely to facilitate the long-term survival of *M. smegmatis*. The findings offer new targets to develop antibiotics against persisters. Further, investigating the corresponding genes in *M. tuberculosis* may provide valuable leads in improving the treatment of chronic and persistent tuberculosis infections.

KEYWORDS

persisters, *Mycobacterium smegmatis*, antibiotic tolerance, biofilm formation, *in vivo* survival

Introduction

Mycobacterium tuberculosis, the etiologic agent of tuberculosis, is reported to infect 2.6 million people in India alone (World Health Organization, 2021). Majority of the cases (90%) constitute a latent or persistent form of infection with no apparent clinical signs or symptoms (Fauvart et al., 2011; World Health Organization, 2021) and comprise the transmissible form of infectious bacteria with the ability to reactivate the disease. Tolerance and resistance are other phenotypes that are sometimes confused with the persistence (Brauner et al., 2016; Schrader et al., 2020). The latter population presents a formidable challenge in eradicating the disease (Zhang, 2014). The molecular mechanisms leading to the development of persistence are not fully understood due to several reasons.

They constitute a small, heterogeneous population that is transient in nature and changes with the environment. Secondly, lack of a biological model that can mimic persistence (Zhang, 2014), and finally, due to the redundancy of mechanisms that select persisters. They constitute a subpopulation of bacteria with non-heritable characteristics that enable them to adapt to stressful conditions for long-term survival. They have the same genetic makeup as the drug-sensitive parent population, indicating that bacterial persistence is an epigenetic trait (Bigger, 1944; Keren et al., 2004; Lewis, 2010; Zhang, 2014; Torrey et al., 2016).

Persister cells are classified into three main categories: spontaneous persisters, triggered persisters, and specialized persisters (Urbaniec et al., 2022). Spontaneous persisters are slow-growing cells formed during the logarithmic phase, independent of any environmental trigger. These persisters are pre-existing in bacterial culture. The triggered persisters constitute non-growing cells produced in the stationary phase of growth due to environmental stress and exhibit an extended lag phase upon inoculation into fresh medium (Balaban et al., 2019). The specialized persisters evolve in response to specific antibiotics (Urbaniec et al., 2022). In *Escherichia coli,* tolerance to fluoroquinolones occurs by extension of the lag phase through growth-inhibiting toxin components (MazF or HipA) of the toxin-antitoxin (TA) systems (Mok and Brynildsen, 2018). Similarly, exposure of *E. coli* to ciprofloxacin reduces the proton motive force (PMF) and ATP levels in the cell, which decreases antibiotic transport, inducing drug-specific persistence (Dörr et al., 2009). In mycobacteria, variation in the levels of the catalase-peroxidase enzymes, necessary for activation of isoniazid, causes a reduction in the effective concentration of the drug, leading to drug-specific persistence (Wakamoto et al., 2013).

Biofilm formation by pathogens has emerged as one of the essential attributes contributing to the establishment of persistent infections in the host (Helaine et al., 2014). The shreds of evidence from *in vitro* experiments revealed an abundance of persisters in the biofilms; a link between the two is established, suggesting a significant role in relapses and recalcitrance of pathogenic bacteria (Keren et al., 2004; Lafleur et al., 2010; Conlon et al., 2013). The presence of persisters is believed to be responsible for antibiotic treatment failure (Van den Bergh et al., 2016; Levin-Reisman et al., 2017). It is widely accepted now that the underlying reasons behind the emergence of antibiotic-tolerant or persister cells are most likely multifactorial, especially within the host, where different interconnected metabolic processes influence each other.

Persisters have been identified in the host-dependent bacterial species, e.g., *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, *Streptococcus suis*, *E. coli*, and *M. tuberculosis*, etc., (Helaine et al., 2014; Michiels et al., 2016). To counter host defense mechanisms, *M. tuberculosis* is reported to express various phenotypes such as biofilm growth (Chakraborty et al., 2021), tolerance to antibiotics (Ciofu et al., 2017), granuloma formation (Barry et al., 2009), survival in the host macrophage (Helaine et al., 2014), and immune evasion (Domingue et al., 2020). The hostile and restrictive environments encountered within the host macrophages and granulomatous lesions force manifestation of phenotypic heterogeneity, which enables a small population to survive/persist longer (Helaine et al., 2014; Mouton et al., 2016; Fisher et al., 2017). *In-vitro* models simulating the stress conditions experienced by mycobacteria inside the host were shown to change the metabolic state of the bacteria and generate a quiescent reservoir of bacterial cells promoting latent infection (Zhang, 2014; Lipworth et al., 2016; Joshi et al., 2021).

To identify genes regulating persistence in different bacterial species, mutagenesis is employed, which results in either decreased (e.g., *ybaL, relA, phoU, sucB, ubiF*, and *lamA*) or increased (e.g., *hipA, metG, tktA, glpD*, and *hupB*) survival propensity (Li and Zhang, 2007; Ma et al., 2010; Rego et al., 2017; Hingley-Wilson et al., 2020). These genes regulate various bacterial pathways such as TA systems, SOS response, signalling pathways, stringent response, antioxidant defence, alternative energy production, enhanced efflux or transporter activity, and phosphate metabolism, etc., (Zhang, 2014). Apparently, persisters are formed through independent parallel mechanisms, providing a significant adaptive advantage to the organism. This redundant design works in favor of the organism, as no single compound can abolish persister formation.

Latency or persistence is frequently observed in pathogenic and non-pathogenic species of mycobacteria. The fast-growing, non-pathogenic *Mycobacterium smegmatis*, which shares 70% homology with the pathogenic *M. tuberculosis*, is often used as a model to study persistence genes (Malhotra et al., 2017; Ranjitha et al., 2020). The present study is an attempt at enhancing our understanding of mycobacterial persistence by screening a transposon mutant library of *M. smegmatis* mc^2155 strain using three key phenotypes commonly associated with persistence - antibiotic tolerance, biofilm formation, and survival in macrophages to identify genes that influence the survival of the bacteria. Our findings have revealed novel targets, which might play a role in the adaptation of *M. smegmatis* leading to persistence.

Materials and methods

Bacterial strains, media, growth conditions, and plasmids

The *M. smegmatis* mc^2155 strain was used to construct and screen a transposon library. Cultures were grown in Middlebrook 7H9 liquid Broth or 7H10 Agar (Difco) solid medium enriched with 10% oleic acid-albumin-dextrose-catalase (OADC) supplement, glycerol (0.5%), and Tween-80 (0.05%). *E. coli* DH5α λ *pir* strain was used for all the cloning experiments and as the host for sequencing the transposon insertions. The chemicals used in the study were purchased from Sigma-Aldrich. A stock of antibiotics was prepared (Andrews, 2001), and dilutions were made using a 7H9 broth medium. All the strains were cultured in triplicate, for each experiment.

Determination of minimum inhibitory concentration of antibiotics

The MIC was determined using resazurin end point microtiter assay (REMA) (Taneja and Tyagi, 2007) with some modifications. The culture was grown till the mid-exponential phase (OD ~ 0.6−0.8) and diluted 1:100 for the assay. The initial dilutions of the antibiotics were prepared in either DMSO or sterile deionized water, and successive 2-fold dilutions were done in 100 µl of

7H9 medium supplemented with 0.05% glycerol (without Tween-80) in the microtiter plates. Antibiotic concentrations ranged from 0.005 to 4 μg/ml. In a total volume of 200 μl, each well contained 100 μl of culture containing 1 x 10^4 CFU and 100 μl of medium containing the antibiotic. A positive control containing bacteria alone and a negative antibiotic control without culture was used to determine the bacterial viability. The plates were sealed with Parafilm and incubated at 37°C for 1−2 days. After 2 days, 30 μl of 0.02% resazurin and 12.5 μl of 20% Tween-80 were added to each well. The colour of the medium changed from blue to pink after 2 days. The fluorescence was determined using a spectrofluorimeter by excitation at 530 nm and emission at 590 nm. The lowest concentration of the antibiotic causing 90% inhibition was considered as the MIC. The percentage inhibition of viability was calculated as shown below:

Viability inhibition (%) = 1-(test well fluorescence/mean fluorescence of positive control) x 100

The MIC was tested on solid media also. A standard culture (1 x 10^4 CFU) was plated onto antibiotic-containing (ranging from 0.005 to 4 μg/ml) and antibiotic-free control 7H10 agar plates in duplicate. The culture was spread with a sterile spreader, sealed with Parafilm, and incubated at 37°C for 3 days. The number of CFUs was recorded after 3 days. The number of live bacteria (CFU) was counted to calculate the MIC.

Antibiotics selection for tolerant cell generation in the wild-type *M. smegmatis*

Tolerant cells generated by wild-type (WT) *M. smegmatis* were evaluated by replica plating the colonies obtained after exposure of the culture to specific antibiotics. *M. smegmatis* was treated with the antibiotics at 10X and 50X MIC (minimum inhibitory concentration), isoniazid (25 and 125 μg/ml), rifampicin (6.25 and 31.25 μg/ml), levofloxacin (0.78 and 3.90 μg/ml), and moxifloxacin (0.039 and 0.195 μg/ml) for 3 days. Samples were taken at different stages of growth, and plated on 7H10 agar medium. The colonies obtained in the previous step were replica plated with and without the drug to obtain the number of tolerant cells. The difference between the total number of colonies and the colonies able to grow in the presence of the antibiotic on sub-culturing were considered as the tolerant population for plotting a time-kill curve.

Construction of transposon mutant library

A mutant library of *M. smegmatis* mc^2155 was constructed by random transposon insertion mutagenesis using a φMycomarT7 phage carrying *Himar1* transposon. The transposon was transferred to *M. smegmatis* by transduction according to Lamichhane et al. (2003) with minor changes – *M. smegmatis* was grown in 7H9 containing ADC to OD_{600} of 1.2 (Lamichhane et al., 2003). The bacterial cells were centrifuged, washed two times in mycobacteriophage (MP) buffer, and the pellet was resuspended in 8 ml of the MP buffer. An aliquot was saved and used as a control for CFU counts. The cell suspension was pre-warmed to 37°C in a water bath and was mixed with approximately 2 ml of 1 x 10^{11} PFU (Plaque forming unit) or MP buffer control. The mixture was plated onto 15 cm 7H10-OADC agar plates (five to ten in number) containing 0.05% Tween-80 and 25 μg/ml kanamycin and incubated at 37°C for 2−3 days. The titer of transduction in *M. smegmatis* was ∼1.36 x 10^5 kanamycin-resistant colonies. After harvesting the mutant colonies from the plates, they were resuspended in 7H9-OADC media containing Tween-80 (0.05%) and glycerol (15%). For storage, the cells were resuspended by sonication in water baths for two cycles of 5 s each and stored in aliquots of 1 ml at −80°C. Transposon insertion was checked by colony PCR, using forward and reverse primers (**Table 1**).

Selection of transposon mutants

A glycerol stock of the mutant library was inoculated in a 7H9 medium supplemented with kanamycin (25 μg/ml), grown at 37°C for 24 h, and spread on 7H10 agar plates. Based on their size, the colonies were classified as small, medium, and large. In the initial elimination stage, the segregated colonies were screened based on biofilm formation and antibiotic tolerance as follows. The colonies were grown in OADC supplemented 7H9 medium containing kanamycin in 96-well microtiter plates at 37°C for 2 days. The cultures were diluted 100 times into fresh 7H9 medium in 96-well plates. The plates were incubated at 37°C for 3 days till the OD_{600} reached the stationary phase. Levofloxacin was added to each well at a concentration of 3.9 μg/ml (50X), and incubation continued. Growth was monitored by measuring OD_{600} on days 3 and 6 and simultaneously streaked on 7H10 supplemented agar plates with no antibiotic to observe the extent of growth inhibition. The antibiotic tolerance assay was repeated with a second fluoroquinolone antibiotic, moxifloxacin. The mutants showing higher susceptibility to both levofloxacin and moxifloxacin after 3 or 6 days of exposure were selected. The antibiotic exposure was repeated to confirm the sensitivity of the mutant phenotype. Next, the selected mutants from the previous step were subjected to two cycles of biofilm formation as the second selection criteria. Those showing a defect in both criteria were subjected to the next round of tests.

Identification of transposon insertion sites

Genomic DNA was digested using *SacII* restriction enzyme, and the fragments were self-ligated as plasmid for cloning. The competent cells of *E. coli* DH5α λ *pir* strain were transformed with the ligated mixture and spread on LB agar plates containing 50 μg/ml of kanamycin. The plates were incubated overnight at 37°C, and individual colonies were grown in 5 ml LB media with shaking at 50 μg/ml kanamycin overnight. Plasmid DNA was isolated from the cultures and sequenced with a primer designed to anneal with the 3' end of the *aph* gene cassette in the transposon to determine the insertion site (**Table 1**). BLAST was used to identify

TABLE 1 Bacterial strains and plasmids were used in this study.

	Relevant characteristics	Sources
Bacterial strains		
M. smegmatis mc^{2}155	*ept-1*, efficient plasmid transformation mutant of mc^{2}6	ATCC
E. coli DH5α λ *pir*	supE44 ΔlacU169 (φ80ΔlacZM15) hsdR17recA1	DF/HCC DNA Resource CORE at Harvard Medical School
Transposon vector		
?MycomarT7	T7 promoter, λ pir+ OriR6K, Kanamycin resistance gene, C9 Himar1 transposon	(Sassetti and Rubin, 2003)
Primers		
Primers used for colony PCR		
kan-Fp	5′-ATGATTGAACAAGATGGATT-3′	Eurofins
kan-Rp	5′-TCAGAAGAACTCGTCAAGAA-3′	Eurofins
Primer used for sequencing of transposon insertion site		
aph-Fp	5′-CCTTCTATCGCCTTCTGTGAGT-3′	Eurofins
Primers used for amplification of transposon disrupted genes with their promoter sequence		
4044pMV261Fp	5′-ATAGGATCCGTACGTATTTGGAGG-3′	Eurofins
4044pMV261Rp	5′-ATAAAGCTTTCACGCCTGTTCACC-3′	Eurofins
2263pMV261Fp	5′-ATAGGATCCCCTACGCCCTG-3′	Eurofins
2263pMV261Rp	5′-ATAAAGCTTTCACACCATCCCGTT-3′	Eurofins
3194pMV261Fp	5′-ATAGGATCCGGCGAAATGCCAGTA-3′	Eurofins
3194pMV261Rp	5′-ATAAAGCTTTTACAGGGTGGCGT-3′	Eurofins
3455pMV261Fp	5′-ATAGGATCCGCACAGCTACCG-3′	Eurofins
3455pMV261Rp	5′-ATAAAGCTTTCAGATCTGACCGGA-3′	Eurofins
0719pMV261Fp	5′-ATAGGATCCCTGCCCCAGG-3′	Eurofins
0719pMV261Rp	5′-ATAAAGCTTTCACCGGGCGGTGA-3′	Eurofins
6655pMV261Fp	5′-ATAGGATCCTATCTGGAGCCCTT-3′	Eurofins
6655pMV261Rp	5′-ATAAAGCTTTCAGCCCCAAAC-3′	Eurofins
0392pMV261Fp	5′-ATAGGATCCGCTTCCCCTCGA-3′	Eurofins
0392pMV261Rp	5′-ATAAAGCTTTCACATCGCCGCT-3′	Eurofins
6145pMV261Fp	5′-ATAGGATCCGCCTGTCCT-3′	Eurofins
6145pMV261Rp	5′-ATAAAGCTTCTACACGCCCT-3′	Eurofins
3233pMV261Fp	5′-ATAGGATCCCTCGGCGTCTTC-3′	Eurofins
3233pMV261Rp	5′-ATAAAGCTTCTAGTAGGCGAACGAC-3′	Eurofins
2211pMV261Fp	5′-ATAGGATCCCGTGACGTCGAT-3′	Eurofins
2211pMV261Rp	5′-ATAAAGCTTTCAGCGCAGGGC-3′	Eurofins

the genes disrupted by insertion, with *M. smegmatis* mc^{2}155 as the reference genome.

Preparation of plasmids for genetic complementation

For complementation, plasmids were constructed by amplifying the selected genes with promoter sequence using *M. smegmatis* genomic DNA as a template with specific primers containing a 5'-BamHI restriction site and a HindIII restriction site at the 3' end. The amplified products were digested with BamHI and HindIII restriction enzymes and ligated in pMV261 vector. The plasmids were electroporated in the respective mutant strains of *M. smegmatis*. The WT strain containing the pMV261 plasmid alone was used as a control.

Growth determination

M. smegmatis and its transposon mutants were grown in triplicate in standard 7H9 liquid medium, and samples were removed at regular intervals for measuring the absorbance at 600 nm. The data presented are the mean values from three individual experiments with standard deviations.

Influence of antibiotic time and dose on tolerance development

The strains were grown in the presence of inhibitory concentrations of levofloxacin and isoniazid and variation in the number of live/tolerant cells with time was measured. The primary cultures were grown in 10 ml of 7H9 medium containing

kanamycin (25 μg/ml) at 37°C for 3 days with shaking at 150 rpm. The cultures were diluted 1:100 in 10 ml 7H9 medium and allowed to grow with shaking until their OD_{600} reached ~ 1.8. These growth conditions were adopted for all the following experiments. An aliquot was removed to determine the CFU as a zero time point of untreated bacterial cultures. 10X MIC of levofloxacin (0.78 μg/ml) and isoniazid (25 μg/ml) were added to all the *M. smegmatis* variant strains and incubated for 6 days at 37°C with shaking. For CFU counts, aliquots were removed every 24 h and plated on 7H10 agar plates supplemented with OADC. The live cells were replica plated with and without the respective antibiotics on 7H10 agar plates and incubated at 37°C.

In another experiment, the WT *M. smegmatis*, the mutants, and their complemented strains were grown in triplicate to stationary phase, as described above. An aliquot was plated to record the CFU count of the untreated cultures. To measure the dose dependence of the strains, levofloxacin and isoniazid were added at different concentrations (10X, 20X, 30X, 40X, and 50X MIC) to all the *M. smegmatis* cultures. After incubation for 3 days at 37°C, the cultures were harvested and plated after serial dilution in 7H10 agar plates supplemented with OADC. The live cells were replica plated with and without antibiotics as described above, and the CFU counts were recorded.

Biofilm formation assay

To examine biofilm formation, *M. smegmatis* strains were cultured in Sauton's medium (without Tween-80) (Ojha et al., 2008). 10 μl of 3 days saturated cultures were inoculated in 1 ml of the medium and grown in 24-well, flat bottom culture plates at 37°C under stationary conditions. After 4 days of incubation, the medium and the non-adherent bacteria were removed carefully by suction, and the wells were washed three times with sterile distilled water. The attached bacteria forming the biofilm were stained for 30 min with 500 μl of 1% crystal violet (CV) at room temperature, rinsed three times with distilled water, and allowed to air dry. The stained biofilm was dissolved in 500 μl of 95% ethanol at 37°C for 30 min and used to measure optical density at 595 nm in an ELISA plate reader (TECAN, Sunrise AZreader). The assay was repeated at least three times with all the strains.

Intracellular survival of the *M. smegmatis* variants in murine macrophages

To measure the rate of clearance of the mutants in cultured macrophages, RAW 264.7 macrophages were seeded at a density of 1 x 10^5 cells per well in 24-well culture plates and incubated for 24 h. Mid-logarithmic phase bacterial strains were centrifuged, rinsed in PBS twice, and resuspended in DMEM containing 10% FBS medium to achieve OD_{600} ~0.1. The bacterial suspension was kept in an ultrasonic water bath for 15 min to disaggregate the clumps and used to infect the macrophages at an MOI (multiplicity of infection) of 25:1. After 2 h, the supernatant containing floating bacterial cells was removed, and the wells were rinsed with fresh medium. Gentamicin (100 μg/ml) was added to each well and incubated for 1 h to kill bacteria attached to the surface. The infected murine macrophages were removed at different time points (0, 24, and 72 h), rinsed with PBS, and lysed in autoclaved water containing 0.1% Triton X-100. The bacterial cells in the lysate were serially diluted in PBS, followed by plating on a 7H10 agar medium enriched with OADC. The plates were incubated for 3 days at 37°C. The assay was repeated at least three times.

Stress tolerance of *M. smegmatis* variants under *in vitro* conditions

To measure the ability of the mutant strains to tolerate stress conditions encountered in the host, the following tests were performed. Tolerance to oxidative stress was evaluated by treatment with hydrogen peroxide. The *M. smegmatis* strains were grown in supplemented 7H9 media till the stationary phase as described above. Fresh 50 ml media were inoculated with the corresponding stationary phase cultures and incubated at 37°C for 3 days. Aliquots of 5 ml were removed at 24 h intervals and treated with 10 mM hydrogen peroxide at 37°C for 2 h. An untreated culture was run in parallel as a control for comparison. At each time point, the cultures were plated and CFUs were determined.

For hypoxic stress, the *M. smegmatis* strains were grown in 10 ml 7H9 medium in glass tubes and incubated at 37°C with shaking at 150 rpm till the OD_{600} reached ~ 0.6—0.8. The cultures were diluted 100 times in 10 ml 7H9 medium in screw-capped, flat-bottom culture tubes with 5 ml ambient air (using a headspace ratio of 0.5). Methylene blue at a concentration of 1.5 μg/ml was added to the medium before sealing to measure oxygen consumption. The culture tubes were sealed well to make them airtight and incubated with shaking at low speed at 37°C for 10 days. The depletion of oxygen was observed with the decoloration of methylene blue. Cells were harvested, plated, and the survival of the cells was determined by CFU counting.

Growth and survival of *M. smegmatis* transposon mutants in mice

Female C57BL/6J mice were sourced from Rodent Research India Pvt. Ltd (Haryana, India). The mice were housed in sterile autoclaved micro-isolator cages maintained at temperature (26°C), light & dark cycles (12 h each), and constant humidity (30%). The animals were provided with free access to food and water during the study. The Institutional Animal Ethics Committee and Biosafety Committees of Jawaharlal Nehru University, New Delhi, India, authorized the protocols. All the experiments were performed following the animal welfare regulations of the World Organization for Animal Health. Qualified personnel performed all experiments in a biosafety level II (BSL-II) facility at the School of Biotechnology, Jawaharlal Nehru University.

To evaluate the *in vivo* survival of the WT, the transposon mutants, and their complemented strains, 6-week-old female C57BL/6J mice (three per group) were used. All the bacterial strains were grown at 37°C with shaking for 2 days, harvested, and resuspended in 0.05% PBST. The mice were injected with 1 x 10^6 CFUs of the *M. smegmatis* strains intravenously via the tail vein. At each time point (1, 4, 7, 14, and 21 days), the mice

were euthanized, and the lungs and spleen were removed aseptically and homogenized in sterile 0.05% PBST. The tissue homogenates were serially diluted in PBST and grown on 7H10 agar plates supplemented with 10% OADC and 25 μg/ml kanamycin. The plates were incubated at 37°C, and bacterial colonies were counted after 3−4 days.

Statistical analysis

All the experiments in this study were repeated at least three times. Graphs were prepared using Graph Pad Prism 8 (GraphPad Software, La Jolla, CA). In the graphs, the data points represent the average of three independent experiments, while the error bars represent the standard deviations. Appropriate statistical tests (i.e., One-way ANOVA and Two-way ANOVA) were performed for multiple comparisons, and a P-value of $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, and $^{****}P < 0.001$ was considered statistically significant.

Results

Generation and screening of transposon mutants

A library of 1.36 x 10^5 transposon mutants of the *M. smegmatis* mc^2155 strain was generated by random insertion of *Himar1* transposon present in φMycomarT7 phage. The *Himar1* transposon requires TA dinucleotide sites in the genome for insertion. A total of 1.36 x 10^5 random transposon mutants generated in this study suggested an approximate 19-fold targeted transposon insertions at 4.89-$\log_{10}$ TA sites (Foreman et al., 2020), in 6938 ORFs present in the genome of *M. smegmatis*. This indicated that the constructed library is likely to be saturated. From the library, 10,000 *aph*-positive clones were screened, and ∼20 were randomly checked by colony PCR for *aph* gene (data not shown). Further screening was based on the chosen phenotypes, such as antibiotic tolerance and biofilm formation. Of the total number of selected mutants, 134 showed reduced tolerance to levofloxacin and moxifloxacin, while 107 mutants were defective in biofilm formation, and ten mutants common in the above two groups, were defective in both the phenotypes. Since the probability of persistence with a defects in two of the typical features is likely to be higher, the latter were selected for detailed studies.

Evaluation of tolerance to antibiotics in wild-type *M. smegmatis*

To generate the antibiotic sensitivity profile of WT *M. smegmatis* to select drugs for later experiments, multiple antibiotics (two first-line anti-TB drugs, isoniazid and rifampicin, and two second-line drugs, levofloxacin and moxifloxacin) were tested. After exposure of the culture to the antibiotics, response of the strain was evaluated by plating on 7H10 agar medium. Next, the colonies obtained in the previous step were replica plated to determine the number of tolerant cells. *M. smegmatis* cells that survived the initial exposure to isoniazid and rifampicin were less sensitive, as the number of live cells remained relatively high after 3 days (**Supplementary Table 1**). However, in the case of isoniazid, the percentage of resistant cells was relatively much smaller than the tolerant population, while treatment with rifampicin produced 80−90% of resistant cells in all the growth phases (**Supplementary Table 1**). Levofloxacin and moxifloxacin antibiotics were more effective in bacterial killing, but like isoniazid, they resulted in a higher proportion of tolerant than resistant cells (**Supplementary Table 1**). The number of surviving bacteria at 10X or 50X MICs was almost similar in all the antibiotics, suggesting that the peak antibiotic sensitivity was attained at 10X level (data not shown). Based on the above observations, isoniazid and levofloxacin at 10X MIC were selected for screening of the transposon mutants.

Identification of genes involved in defective phenotypes

DNA sequencing of the mutants revealed the genes disrupted by transposon insertion (**Table 2**). The information on gene length, its location within the genome, and the putative functional product of the identified genes are shown in **Table 2**. Mutant strains C1, with phenotypes (antibiotic resistant, biofilm negative), C2 (antibiotic resistant, biofilm positive), and C3 (antibiotic sensitive, biofilm positive) were used as controls in the stress endurance assays. Some of the genes belonged to different pathways, such as biotin biosynthesis, cellular metabolism, glycan modifications, oxidative phosphorylation, etc. The functional aspect of the genes was derived from the KEGG (Kyoto Encyclopaedia of Genes and Genomes) database.

General properties of the selected transposon mutants

To characterize the transposon mutants, the growth profile of the strains was examined, which showed an identical pattern with WT *M. smegmatis* (**Supplementary Figure 1**), and the MIC values of the antibiotics - isoniazid, rifampicin, levofloxacin, and moxifloxacin, were also not significantly different from the parental strain (**Supplementary Table 2**). These results suggest that the reduction in tolerance of the transposon mutants is not due to reduced growth rate or greater antibiotic sensitivity, but independent of the growth phase, antibiotic type, and antibiotic concentration.

Kinetics of variation of antibiotic tolerance with time

To evaluate the antibiotic tolerance of the strains with time, the live cell count of the selected mutants was measured for 6 days in the presence of 10X MIC of the antibiotics. Isoniazid and levofloxacin were used followed by replica plating to exclude the resistant from the tolerant population. A time-kill curve of

TABLE 2 Genes selected through transposon insertion of *M. smegmatis* genome.

Transposon mutants	*M. smegmatis* mc^{2}155	Gene length	Gene location	Functional product	Operonic existence	*M. tuberculosis* orthologs (% identity)	Transposon insertion site
M3	*msmeg_4044*	1788 bp	4115906-4117693	GAF domain containing protein	No	Rv1429 (28%)	1538
M65	*msmeg_2263 (hybC)*	1608 bp	2346098-2347705	Hydrogenase-2, large subunit	No	–	1194
M72	*msmeg_3194 (bioB)*	1062 bp	3273453-3274514	Biotin synthase	No	Rv1589 (87%)	492
M98	*msmeg_3455 (hslR)*	384 bp	3521165-3521548	Ribosome associated heat shock protein 15	No	–	131
L20	*msmeg_0719*	1140 bp	807543-808682	Flavohemoprotein	No	Rv0385 (68%)	319
L40	*msmeg_6655*	777 bp	6709869-6710645	Hypothetical protein	No	–	214
L47	*msmeg_0392*	1515 bp	441687-443201	Putative glycosyltransferase	No	Rv1524 (58%)	686
L67	*msmeg_6145*	717 bp	6213505-6214221	Hypothetical protein	No	–	114
S12	*msmeg_3233 (cydA)*	1464 bp	3316161-3317624	Cytochrome bd ubiquinol oxidase subunit I	Yes	Rv1623c (78%)	617
S14	*msmeg_2211*	840 bp	2291531-2292370	DNA-binding protein	No	–	382
C1	*msmeg_3373*	1272 bp	3443225-3444496	Major facilitator superfamily protein MFS_1	No	–	704
C2	*msmeg_3737*	783 bp	3801921-3802703	Integral membrane protein	No	–	183
C3	*msmeg_3985*	1332 bp	4055023-4056354	Integral membrane transport protein	No	–	148

levofloxacin is shown in Figure 1. Out of the ten mutants, CFU counts in the *msmeg_3233* (*cydA*), *msmeg_0392*, *msmeg_6655*, *msmeg_2211*, *msmeg_0719*, *msmeg_3194* (*bioB*), *msmeg_6145*, *msmeg_3455* (*hslR*), and *msmeg_2263* (*hybC*) strains were 2.19-log, 1.91-log, 1.02-log, 0.84-log, 0.83-log, 0.76-log, 0.76-log, 0.7-log, and 0.66-log, respectively, fold lower than the parental strain. The complemented strains showed the same growth kinetics as the WT strain. Likewise, time-kill curves at 10X MIC of isoniazid after 3 days showed a reduction in the number of live/tolerant cells of 1.97-log, 0.95-log, 0.92-log, 0.89-log, 0.83-log, 0.71-log, 0.71-log, 0.65-log, and 0.43-log, in the *cydA*, *msmeg_0392*, *msmeg_6655*, *bioB*, *hslR*, *msmeg_2211*, *msmeg_6145*, *msmeg_0719*, and *hybC* mutant strains, respectively, compared to the parental strain (Figure 2). No significant difference in the number of live cells was observed in the *msmeg_4044* mutant compared to the parental strain in response to either antibiotic. In most cases, the maximum reduction was recorded on day 3 of incubation of the strains. Taken together, the above results demonstrate that the number of tolerant cells was reduced in all the mutants. The decrease in the number of tolerant cells was significant in *cydA*, *msmeg_0392*, and *msmeg_6655* mutants relative to the WT strain and may play a role in the development of persistence in *M. smegmatis*.

Kinetics of variation of antibiotic dose with tolerance

The number of tolerant cell counts at varying antibiotic concentrations (10X−50X) was measured after 3 days of exposure. The change in the number of live cells with increasing concentrations of levofloxacin is shown in Figure 3. Maximum antibiotic susceptibility with respect to the WT was observed between 10X and 20X MIC in most of the mutants. The fold decrease in the live cell counts of *cydA*, *msmeg_0392*, and *msmeg_6655* mutants was 2.17-log, 2.21-log, and 1.21-log, respectively, relative to the parent strain, over the entire range of antibiotic concentrations (Figure 3). The mutant strains complemented with the respective WT genes showed full recovery

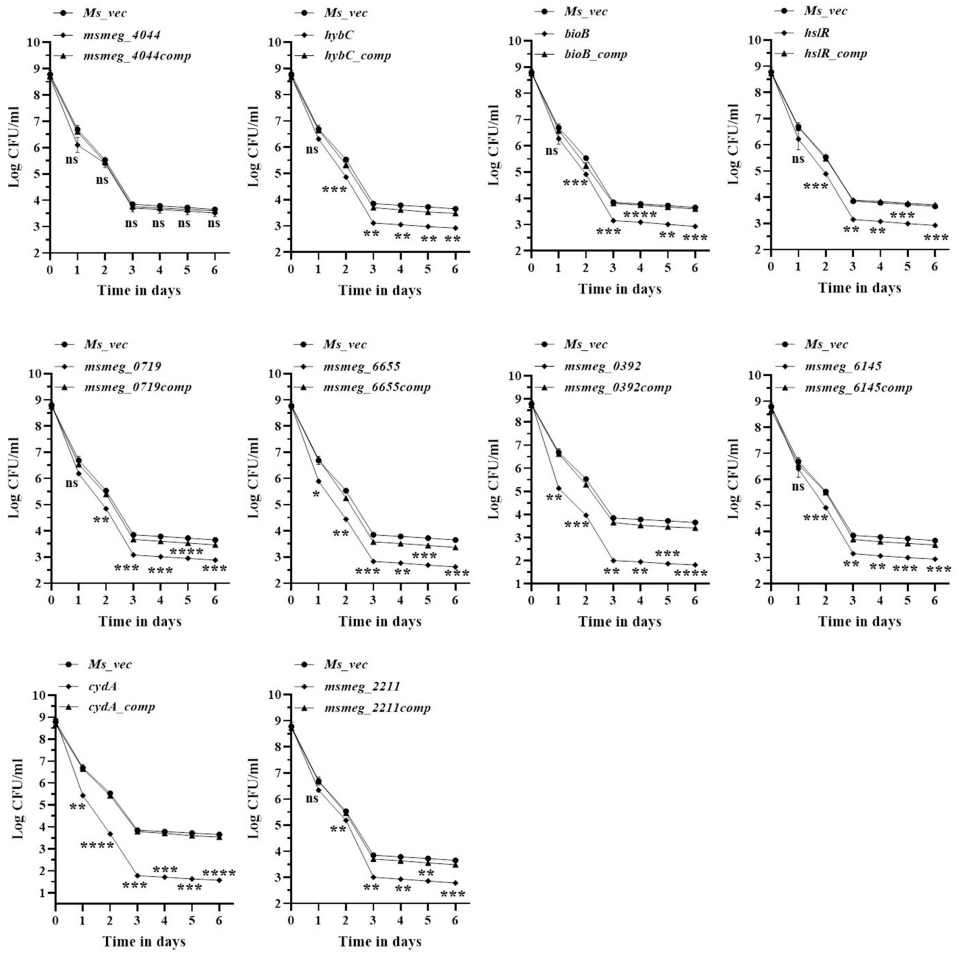

FIGURE 1
Time-dependent tolerance of *M. smegmatis* mutants against levofloxacin. Cultures were grown till OD_{600} ~1.8, and levofloxacin was added. Samples were removed on day 1, 2, 3, 4, 5, and 6 and tolerant cell numbers were obtained by replica plating as described in Methods. The error bars represent the mean ± SD from three independent experiments. The *P*-values were calculated by two-way ANOVA with Dunnett's multiple comparison tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, and ****$P < 0.001$ in all the experiments.

in the number of surviving cells. The reduction in the levels of live cells in the remaining transposon mutant ranged between 0.5-log to 1-log compared to the WT and complemented strains (Figure 3). At higher levofloxacin concentrations, the trend in the sensitivity of the strains followed the same pattern as before. In the case of isoniazid, the maximum reduction in the counts of drug-tolerant cells occurred at 10X MIC in all the mutants, as shown in Figure 4. The *cydA* mutant recorded a maximum reduction of ~ 2-log, while in the remaining mutants, the decrease in the number of tolerant cell counts varied between 0.5-log and 1-log. Further, similar results were obtained at higher concentrations of isoniazid (Figure 4). The sensitivity of *msmeg_4044* mutant strain to both levofloxacin and isoniazid was not significantly different from the WT strain.

Effect on Biofilm formation by *M. smegmatis* strains

The ability to form biofilm is a hallmark of persistent pathogens causing chronic infections. Hence, it was used as a marker to measure tolerance of *M. smegmatis*. Biofilm development on hydrophobic, plastic surface by the mutants is shown in Figure 5. The CV staining confirmed variable degrees of defect in biofilm formation by all the mutants relative to *M. smegmatis* or the complemented strains. The *msmeg_4044*, *bioB*, *msmeg_0719*, *msmeg_6655*, and *msmeg_0392* variants showed a significant reduction in CV binding, while *hybC*, *hslR*, and *msmeg_2211* mutant strains were affected to a lesser degree. The control mutant stains C1 showed ~50% reduction, while C2 and C3 showed no defect in biofilm development, as expected (Figure 5). Our results suggest that the disrupted genes in the selected transposon mutants are directly or indirectly involved in the process of biofilm formation in *M. smegmatis*, eventually helping the cell to survive.

Survival of the mutants in mouse macrophages

To evaluate the ability of the mutants to tolerate the hostile conditions *in vivo*, they were infected in the murine macrophage cell line. The clearance rate of all the mutant strains from the RAW 264.7 macrophages was faster than the WT strain from

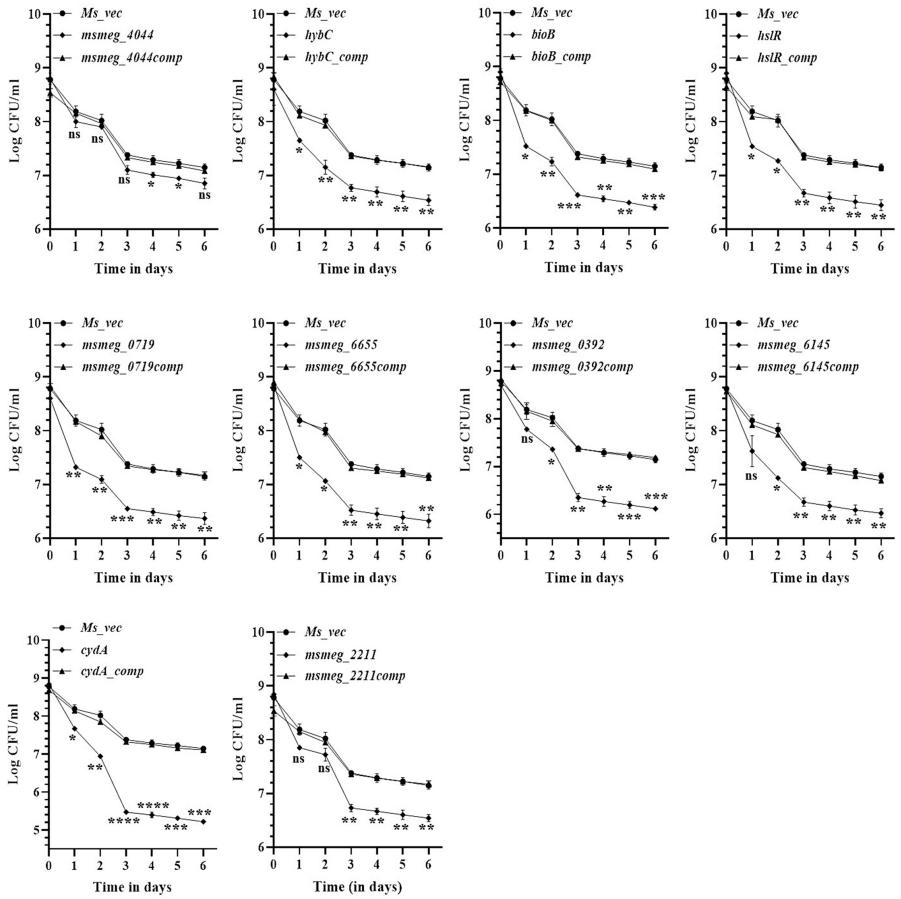

FIGURE 2
Time-dependent tolerance of *M. smegmatis* mutants against isoniazid. Cultures were grown to stationary phase (OD_{600} ~1.8) and isoniazid was added. Samples were removed on day 1, 2, 3, 4, 5, and 6 and tolerant cell numbers were obtained by replica plating as described in Methods. The error bars represent the mean ± SD from three independent experiments. The *P*-values were calculated by two-way ANOVA with Dunnett's multiple comparison tests. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, and *****P* < 0.001 in all the experiments.

24 h onwards. After 72 h, *msmeg_0719* recorded more than 2-log; *msmeg_6655*, *cydA*, *msmeg_4044*, *bioB*, and *msmeg_0392* between 1-log to 2-log; and *msmeg_6145*, *hybC*, *hslR*, and *msmeg_2211* mutants recorded about 0.5-log, lower counts than the WT and the complemented strains (Figure 6). Overall, all ten transposon mutants displayed a variable degree of defect in intracellular survival within the murine macrophages. The clearance time of the control strains C1, C2, and C3 were similar to the WT strain, suggesting that a defect in single phenotype is not enough to cause a significant reduction in the survival of *M. smegmatis*.

Stress response of *M. smegmatis* variants

To examine the stress response of the mutants, the latter were exposed to conditions encountered by mycobacteria during infection, e.g., oxidative stress and hypoxic environment, which reduced the number of survivors in all the mutants compared to the WT strain. All the strains displayed greater sensitivity to oxidative stress at the peak of the growth phase after 48 h. The *msmeg_0719* and *cydA* mutants appear more sensitive than others in the group, showing maximum reduction of ~ 2-log in CFU compared to the WT strain (Figure 7A). While ~ 1 log-fold reduction was observed in the *hybC*, *bioB*, *msmeg_0392*, and *msmeg_6145* mutants over the WT strain (Figure 7A). Of the nine transposon mutants tested, *msmeg_4044*, *hslR*, and *msmeg_6655* mutants, were not affected significantly during exposure to oxidative stress. The number of live cells in the control mutant strains was similar to WT strain, reiterating the synergistic action of multiple genetic loci in adaptation and long-term survival of *M. smegmatis* (Figure 7A).

Some of the mutants showed higher sensitivity to hypoxic stress (Figure 7B). The strain *msmeg_0719* showed maximum reduction of ~1-log in CFU counts, while *hybC*, *bioB*, *msmeg_0392*, *cydA*, and *msmeg_2211* were all affected adversely with lower than 1-log-fold change under hypoxic conditions. As in oxidative conditions, the *hslR* and *msmeg_6655* mutants, were not affected significantly (Figure 7B).

Survival of the transposon mutants in mice

The degree of tolerance of the strains *in vivo* was tested in mice. C57BL/6J mice were injected with *M. smegmatis* strains,

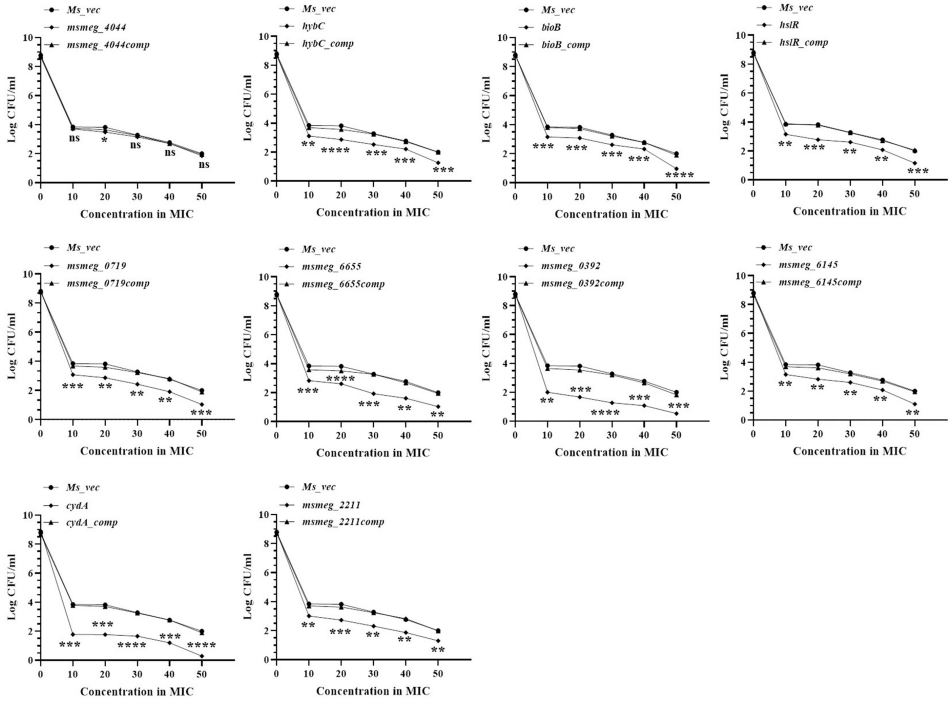

FIGURE 3
Dose-dependent tolerance of *M. smegmatis* mutants against levofloxacin. Stationary phase cultures were exposed to different concentrations of levofloxacin and incubated at 37°C for 3 days. Samples were removed on day 3 and tolerant cell numbers were obtained by replica plating as described in Methods. The error bars represent the mean ± SD from three independent experiments. The *P*-values were calculated by two-way ANOVA with Dunnett's multiple comparison tests. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, and *****P* < 0.001 in all the experiments.

and CFU in the lungs and spleen were enumerated. In general, the bacterial burden was substantially reduced in the organs by 7 days and was fully cleared at 21 days of infection. After 7 days of infection, the bacterial counts in the lungs and the spleen of several mutants, namely *msmeg_0719*, *hybC*, *bioB*, and *cydA*, were ∼1-log lower than the WT and the complemented strains. The CFU counts decreased further at 14 days in the lungs of *msmeg_0719*, *msmeg_0392*, *hybC*, *bioB*, *cydA*, and *msmeg_2211* mutants (ranging between 1.7-log to 0.66-log) with respect to the parent strain, while *msmeg_6145* was not significantly different from the *M. smegmatis* control (**Figure 8A**). Likewise, the number of surviving cells in the spleens of *msmeg_0719*, *msmeg_0392*, *hybC*, *bioB*, *cydA*, and *msmeg_2211*, mutants was 1.52-log, 1.48-log, 1.04-log, 0.97-log, 0.6-log, and 0.53-log lower than the WT and complemented strains after 14 days (**Figure 8B**). Overall, initially (7 days) the rate of clearance of WT bacteria in the spleen was higher (2.35-log) than in the lungs (1.82-log), but at later time point (14 days), the clearance rate was nearly same in both the organs. These results indicate that due to the disruption of some genes, the six mutants mentioned above displayed a significantly lower propensity to survive under *in vivo* conditions than the parental strain, and the genes affected in the latter may play a role in bacterial persistence in the host tissues.

Discussion

It has become abundantly clear that there is no single mechanism to explain the phenomenon of persistence in bacteria (Hu and Coates, 2005; Zhang, 2014; Urbaniec et al., 2022). The ability of *M. tuberculosis* and *M. smegmatis* to form persister cells *in vitro* and *in vivo* has been demonstrated in several studies (Grant et al., 2012; Manina et al., 2015; Torrey et al., 2016). Here, we have adopted three different screening criteria intimately linked to bacterial persistence, e.g., antibiotic tolerance, biofilm formation, and survival *in vivo* in the macrophages, to screen a transposon mutant library of *M. smegmatis* mc^2155. The rationale for using multiple phenotypes for selection was based on the assumption that a gene may control multiple phenotypes indirectly by participating in different metabolic pathways. Hence, disruption of a genetic locus, manifesting defects in multiple phenotypes is likely to be involved in persistence development in a strain. Also, supported by our observations that multiple disabilities in a strain increased the susceptibility of the organism to host clearance mechanisms.

The *msmeg_*3233 gene encodes the CydA subunit of cytochrome bd oxidase. In mycobacteria, the electron transport chain (ETC) terminates through the actions of two-terminal oxidases: an aa3-type cytochrome c oxidase and cytochrome bd oxidase. The latter is involved in the generation of the PMF to fuel energetic processes in the cell membrane (Cook et al., 2014). The cytochrome bd oxidase has been implicated in the adaptation of *M. tuberculosis* to host immunity-induced stress microenvironments during infection (Giuffrè et al., 2012; Forte et al., 2016). In *M. smegmatis*, although not essential under normal oxygen conditions, maintaining mycobacterial respiration is indispensable during hypoxic conditions (Aung et al., 2014; Jeong et al., 2018). In different mycobacterial species, *cydA* expression

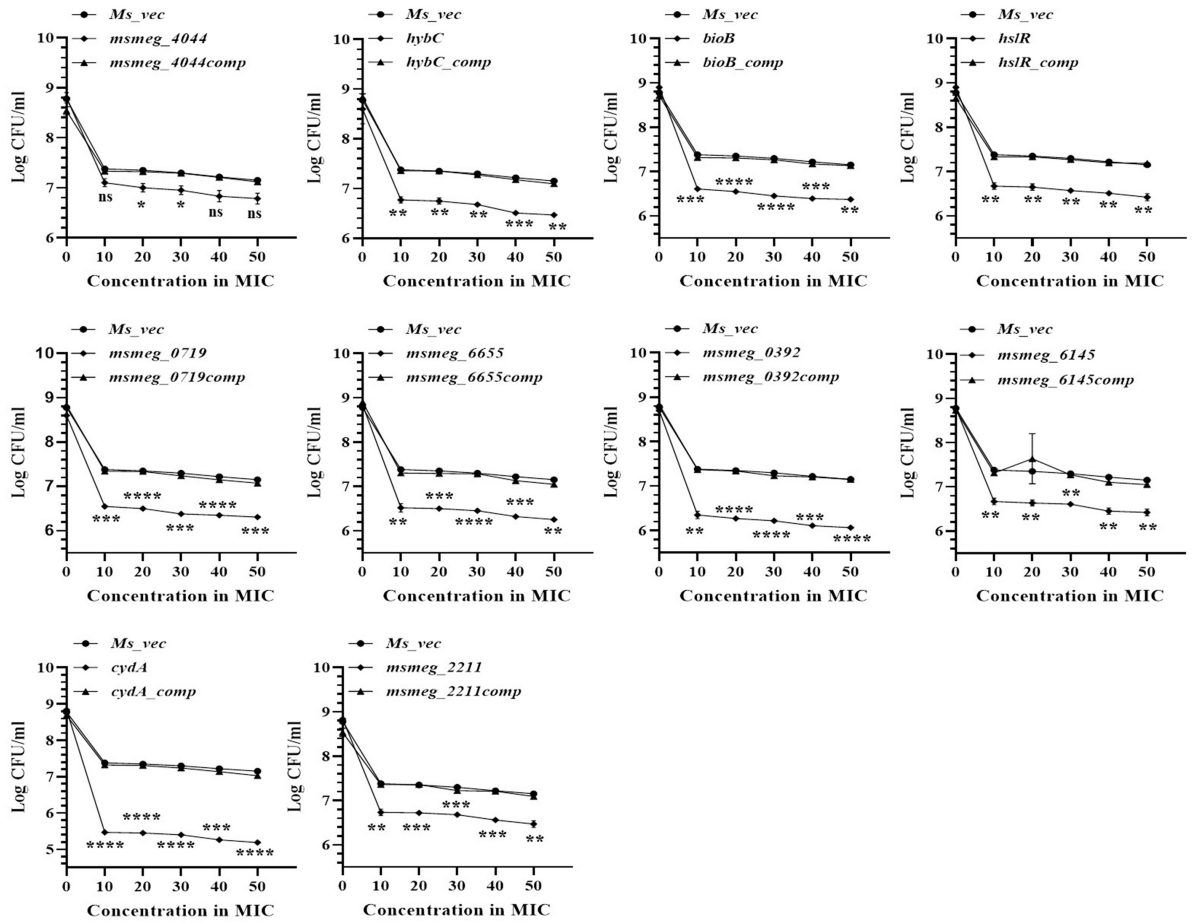

FIGURE 4
Dose-dependent tolerance of *M. smegmatis* mutants against isoniazid. Stationary phase cultures were exposed to different concentrations of isoniazid and incubated at 37°C for 3 days. Samples were removed on day 3 and tolerant cell numbers were obtained by replica plating as described in Methods. The error bars represent the mean ± SD from three independent experiments. The *P*-values were calculated by two-way ANOVA with Dunnett's multiple comparison tests. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, and *****P* < 0.001 in all the experiments.

was induced by cell wall inhibitors, e.g., Q203, bedaquiline, nitroimidazoles, augmentin, potassium cyanide, and clofazimine, emphasizing its importance in stress alleviation and survival (Koul et al., 2014; Boot et al., 2017; Kalia et al., 2017). Based on these reports, cytochrome bd oxidase appears to relieve the pressure on the ETC and allow the bacteria to persist longer by maintaining membrane potential in the absence of cytochrome bcc1-aa3 complex and ATP synthase activities. In the light of previous studies, the defects displayed by *cydA* mutant in all the parameters tested, make a strong case for a key role in global stress management and survival of *M. smegmatis*.

Disruption of the *msmeg_0719* gene is attributed to faster clearance of the mutant, making it sensitive to intracellular stress conditions; the most important among them were oxidative stress, hypoxia, and survival in the mouse macrophages. Oxidative stress is one of the major causes of death of intracellular pathogens. The reactive oxygen species released within macrophages cause lipid peroxidation of the lipids-rich cell walls of mycobacteria and produce toxic methylglyoxal and D-lactate (Rachman et al., 2006). In *M. tuberculosis*, the corresponding ortholog Rv0385 encodes a peripheral membrane-associated flavohemoglobin, which catalyzes D-lactate to pyruvate and protects the respiratory membranes from toxic ROS-mediated killing (Gupta et al., 2012). Secondly, under hypoxic conditions, the heme domain of the protein transfers the electrons acquired from D-lactate to the electron transport chain in the cell membrane to produce energy at a low level, keeping the bacteria viable in a non-dividing state within the host (Gupta et al., 2012). The precise mechanism of Rv0385 in the persistence of *M. tuberculosis* would require further investigation. Since it has been conserved in the genome of a number of the pathogenic and non-pathogenic mycobacterial strains (Gupta et al., 2011), it could be a potential target for combating long-term, non-replicative survival of mycobacteria.

In biological systems, biotin acts as a cofactor for enzymes that catalyze metabolic reactions e.g., membrane lipid synthesis, tricarboxylic acid cycle, and amino acid metabolism etc., (Salaemae et al., 2016). Biotin is synthesized from pimeloyl-CoA using four enzymes encoded by *bio*F, *bioA*, *bioD*, and *bioB* genes organized in an operon in *M. tuberculosis* and in two separate ones in *M. smegmatis* (Tang et al., 2014; Salaemae et al., 2016). *BioB* gene plays a key role in biotin synthesis in both the mycobacterial species (Fan et al., 2015; Lazar et al., 2017). Development of auxotrophy in the *bio*B gene mutant (**Supplementary Figure 2**) and significant reduction in the live cell counts under different stress conditions,

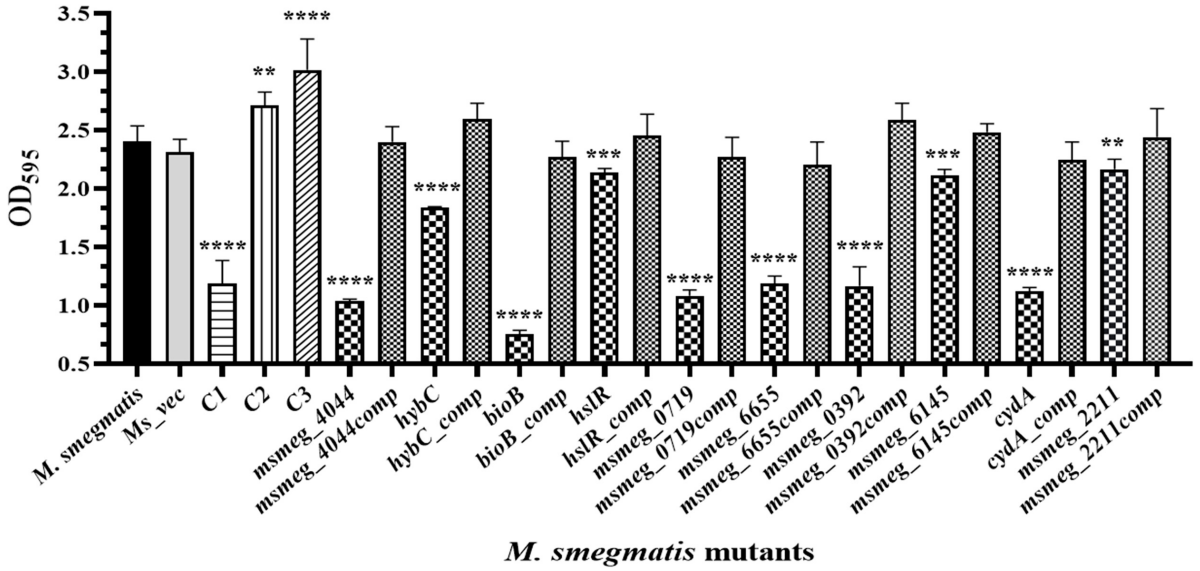

FIGURE 5
Biofilm formation by transposon mutants. Quantitative representation of the biofilm formed by the mutants. The stationary phase saturated culture was inoculated in Sauton's medium as above and incubated at 37°C for 4 days. The medium was removed carefully, and adherent biofilms were stained with crystal violet for optical density measurement at 595 nm. The error bars indicate mean ± SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.005, and ****P < 0.001 in all the experiments.

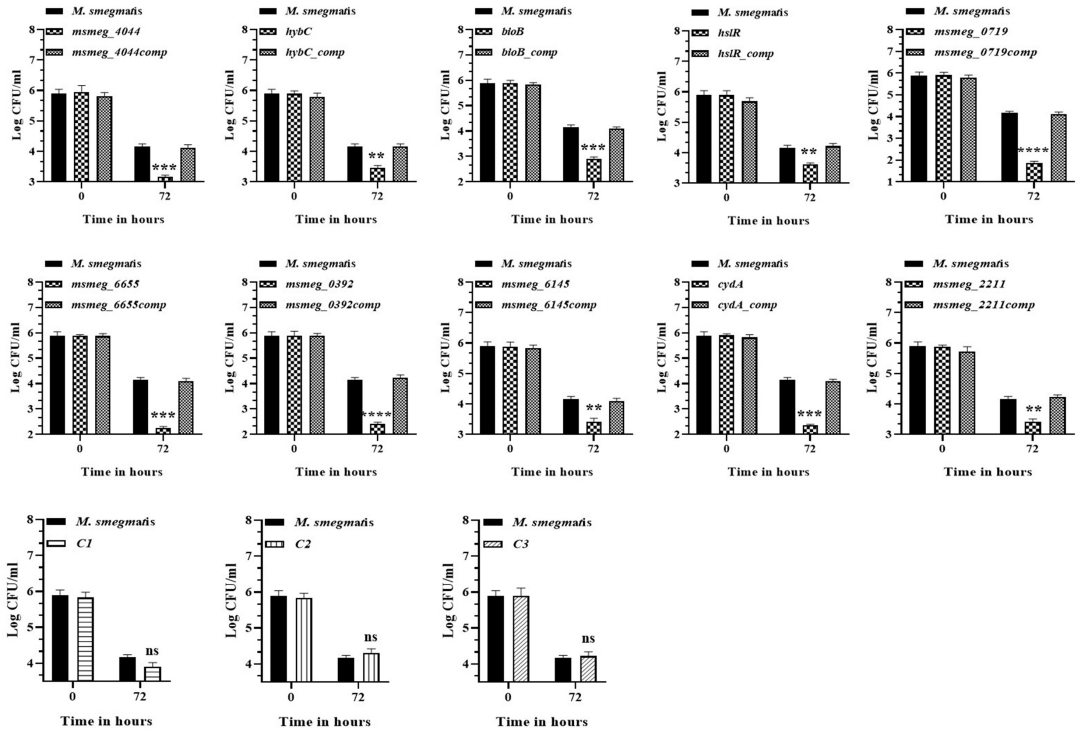

FIGURE 6
Survival of *M. smegmatis* mutants in murine macrophages. Cultured RAW 264.7 cells were infected with *M. smegmatis* strains at m.o.i of 25:1. The cells were harvested at 0, 24, and 72 h post-infection and CFUs counts were obtained by plating appropriate dilutions on 7H10 agar plates supplemented with kanamycin (25 μg/ml). The error bars represent mean ± SD from three individual experiments. The *P*-values were calculated by two-way ANOVA with Dunnett's multiple comparison tests. *P < 0.05, **P < 0.01, ***P < 0.005, and ****P < 0.001 in all the experiments.

e.g., bactericidal antibiotics, oxidative stress, intracellular survival, and hypoxia, reiterate the importance of biotin synthesis in growth and global stress management in *M. smegmatis*.

The reduction in biofilm growth of the *bioB* mutant is most likely a direct effect of disruption in biotin synthesis by the strain. Further, in a *bioB* mutant of *M. bovis*, increased susceptibility

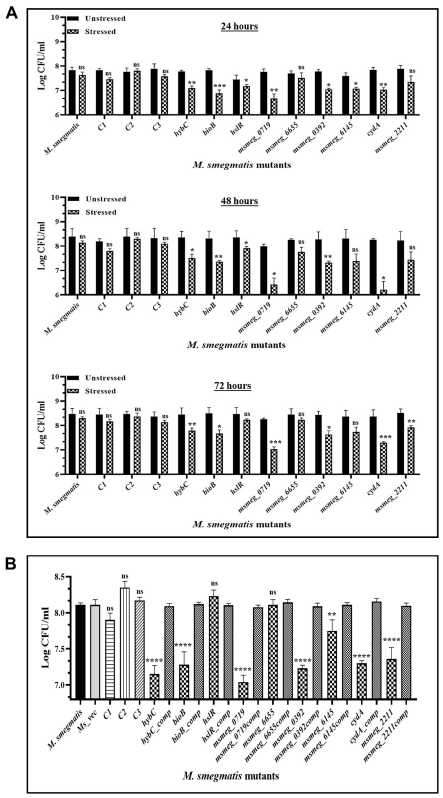

FIGURE 7
Survival of *M. smegmatis* mutants under *in vitro* stress conditions. **(A)** Stationary phase cells of bacterial strains were washed and exposed to 10 mM hydrogen peroxide at 37°C for 2 h at different time intervals. **(B)** All the bacterial strains were incubated in hypoxic conditions for 10 days at 37°C. The CFUs were recorded by plating on 7H10 agar plates supplemented with kanamycin. The error bars represent the mean ± SD from three independent experiments. The *P*-values were calculated by two-way ANOVA with Dunnett's multiple comparison tests. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, and *****P* < 0.001 in all the experiments.

to Para-amino salicylic acid and rifampicin in biotin-limiting conditions was attributed to lack of biotin, implying its significance in antibiotics tolerance in mycobacteria (Howe et al., 2018). Thus, mutation in the *bioB* gene appears to be responsible for the defects in both the phenotypes selected as our screening criteria in *M. smegmatis*. Biotin is known to play a critical role in the biofilm growth of bacteria, which protects them from the hostile conditions in the host (Chakraborty et al., 2021). Ojha and Hatfull (2007) have reported activation of *bioB* gene transcription in the late stages of biofilm formation in *M. smegmatis*. Considering the degree of attenuation of the mutant, a potential role of the *bioB* gene in the slow-growing, non-replicating, persistent state of *M. tuberculosis* cannot be ignored.

The *msmeg_0392* gene encodes a putative glycosyltransferase enzyme involved in carbohydrate transport and metabolism (Vera-Cabrera et al., 2007). It belongs to the Gtf3 family of proteins, necessary for synthesizing glycosylated glycopeptidolipids (GPLs) in *M. smegmatis* (Deshayes et al., 2005). It has been reported that *M. smegmatis* produces triglycosylated GPLs during the late stationary phase under low carbon conditions (Ojha et al., 2002), implicating their role in shielding the cells in unfavorable conditions. Thus, the enhanced susceptibility of the *msmeg_0392* gene mutant to multiple stress conditions merits further investigations to learn about its mechanism of action in the context of persistence. Additionally, the study of *M. tuberculosis* ortholog (*rv1524*) may also reveal necessary attributes for the long-term survival of the pathogen.

The *msmeg_2263* gene is involved in molecular hydrogen (H_2) metabolism in mycobacteria (Berney et al., 2014). The *msmeg_2263* (*hybC*) and *hybA* genes encode the large and small subunits, respectively, of the nickel and iron-dependent hydrogenase-2 enzyme in *M. smegmatis*. The hydrogenases catalyze the conversion of molecular hydrogen into electrons and protons. The protons are responsible for generating the PMF, whereas the electrons enter aerobic or anaerobic respiratory chains (Benoit et al., 2020). The hydrogenases maintain the flow of reducing equivalents to the ETC during conditions of energy limitation. Genetic studies have shown that the *msmeg_2263* gene is induced during starvation and hypoxia, which promotes survival by oxidizing H_2 in *M. smegmatis* (Berney and Cook, 2010; Cook et al., 2014). Further, under hypoxia, mycobacteria switch on hydrogenases to adapt and, like anaerobic bacteria, transfer electrons in the absence of exogenous electron acceptors through the unique electron carriers—ferredoxins (Berney and Cook, 2010). Based on the available information, it is clear that the role of hydrogenases in facilitating long-term survival under low carbon/energy and oxygen conditions is crucial for *M. smegmatis*, making it a good target for preventing persister formation. Furthermore, it would be interesting to identify counterparts of the gene in *M. tuberculosis* and study their role in persistence.

The emerging scenario from this study highlights the cooperative role of (i) alternate routes of energy generation in low carbon and hypoxic situations and (ii) protection from free radicals generated by oxidative burst in the phagocytic cells, as the key factors that enable mycobacteria to outlive the hostile environment in the host. Presently we are unable to propose a mechanism of action of other genes that were picked up by our screen e.g., *hslR*, a ribosome-associated heat shock protein15; *msmeg_6655*, and *msmeg_6145*, hypothetical proteins; *msmeg_2211*, a DNA-binding protein; and *msmeg_4044*, a GAF domain-containing protein in mycobacteria, due to lack of comprehensive information about their functional nature and will be the subject of our future studies.

In comparison to the pathogenic mycobacterial strains, the faster growth rate of *M. smegmatis* may raise questions about its suitability as a model to study tolerance/ persistence. However, the long-term survival of an organism requires multiple attributes other than growth rate to tolerate the hostile host environment. The literature is replete with studies performed in *M. smegmatis*, which provide valuable insight into the unique characteristics of the genus Mycobacterium (Sharbati-Tehrani et al., 2005; Malhotra et al., 2017; Ranjitha et al., 2020; Sparks et al., 2023). Using *M. smegmatis* as a model has the advantage of acquiring a large volume of data in a short time for studying the pathogenic traits of a species. This study was aimed to do the groundwork for identifying potential genes (with orthologs in *M. tuberculosis*) involved in the long-term survival of mycobacteria. We assumed that investigating the conserved stress-inducible housekeeping genes of *M. smegmatis* may reveal information applicable to *M. tuberculosis* persistence.

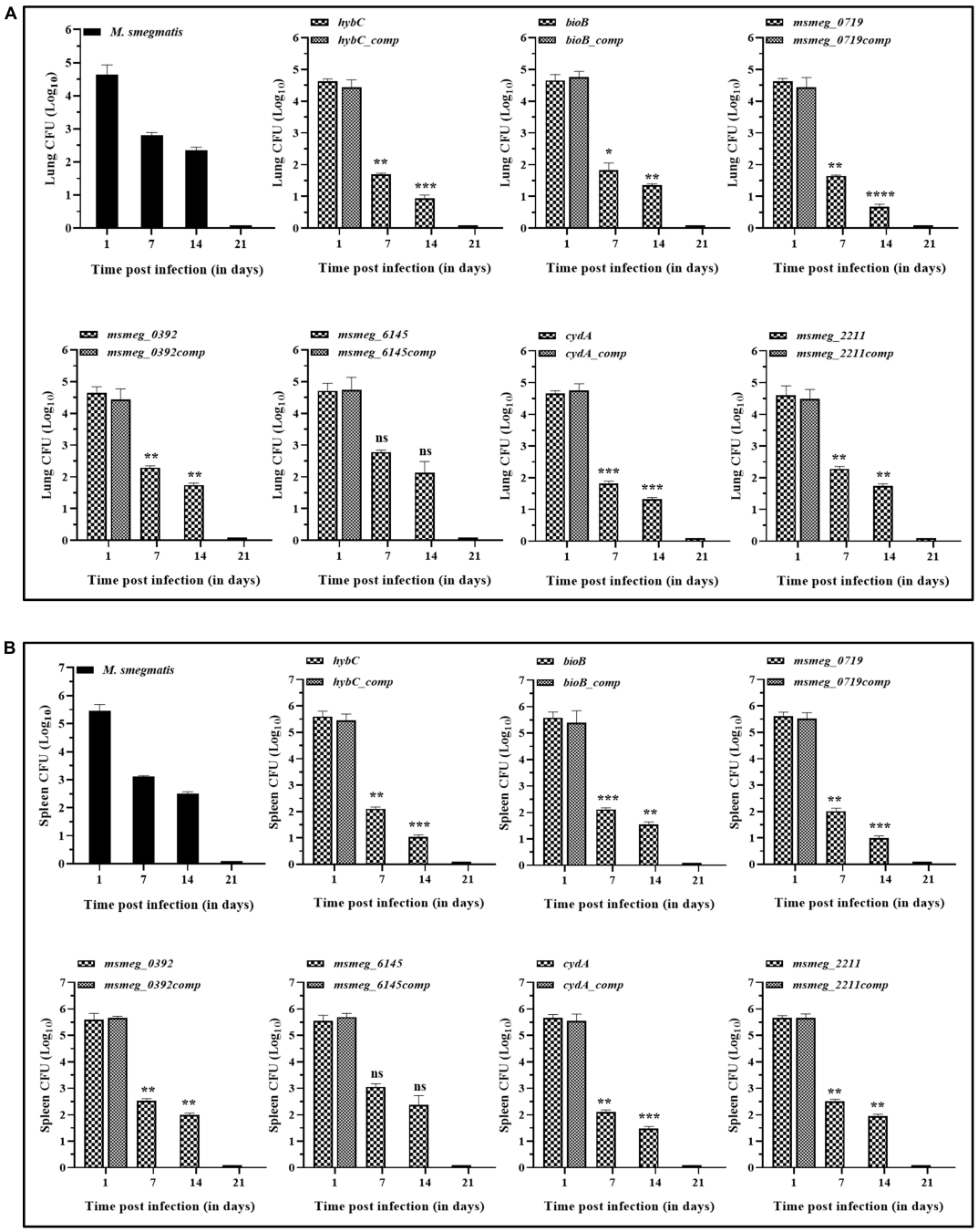

FIGURE 8
Persistence of *M. smegmatis* mutants in lungs and spleen of mice. C57BL/6J mice were injected intravenously with 1 x 10^6 CFUs of actively growing cells. Survival of the bacteria in the **(A)** lungs and **(B)** spleen was determined on day 1, 7, 14, and 21 by plating appropriate dilutions on 7H10 agar plates containing kanamycin (25 μg/ml). At day 21 complete bacterial clearance was observed. The data for complemented strain was plotted for day 1, as it indicates the gene function was restored. The statistical significance was determined by comparing mutant strains with the wild type at different time points of infection. The error bars represent the mean ± SD from three independent experiments. The *P*-values were calculated by two-way ANOVA with Dunnett's multiple comparison tests. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, and *****P* < 0.001 in all the experiments.

It is challenging to identify genes involved in persistence development due to the transient nature of the phenotypes and the redundancy of mechanisms promoting long-term survival. Each effort reveals new targets, providing insight to understand the process. Likewise, this study has identified some target genes in *M. smegmatis*. To our knowledge, the five genes have not been described in the context of the persistence of mycobacteria. These genes participate in vital metabolic pathways, including alternate methods of energy production, hydrogen metabolism, biotin synthesis, biofilm formation, and carbohydrate metabolism. Some of these genes have orthologs in *M. tuberculosis* and offer possibilities for developing novel antibiotics to treat chronic and persistent tuberculosis infections.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the Institutional Animal Ethics Committee of Jawaharlal Nehru University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HJ: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. DK: Conceptualization, Formal Analysis, Methodology, Writing – review & editing. SM: Supervision, Visualization, Writing – review & editing. RB: Funding acquisition, Resources, Supervision, Visualization, Writing – review & editing. NB: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Supervision, Writing – review & editing.

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Conflict of interest

NB and DK were employed by the Divacc Research Laboratories Pvt 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1302883/full#supplementary-material

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Understanding bacterial pathogenicity: a closer look at the journey of harmful microbes

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Bacteria are the most prevalent form of microorganisms and are classified into two categories based on their mode of existence: intracellular and extracellular. While most bacteria are beneficial to human health, others are pathogenic and can cause mild to severe infections. These bacteria use various mechanisms to evade host immunity and cause diseases in humans. The susceptibility of a host to bacterial infection depends on the effectiveness of the immune system, overall health, and genetic factors. Malnutrition, chronic illnesses, and age-related vulnerabilities are the additional confounders to disease severity phenotypes. The impact of bacterial pathogens on public health includes the transmission of these pathogens from healthcare facilities, which contributes to increased morbidity and mortality. To identify the most significant threats to public health, it is crucial to understand the global burden of common bacterial pathogens and their pathogenicity. This knowledge is required to improve immunization rates, improve the effectiveness of vaccines, and consider the impact of antimicrobial resistance when assessing the situation. Many bacteria have developed antimicrobial resistance, which has significant implications for infectious diseases and favors the survival of resilient microorganisms. This review emphasizes the significance of understanding the bacterial pathogens that cause this health threat on a global scale.

KEYWORDS

bacteria, host susceptibility, immune response, AMR, disease severity

Introduction

"The enemy was the microbial world, and over the centuries, it has killed more people than all of man's wars combined."

—Tess Gerritsen, Gravity

Microbes are tiny and trillion can make us sick or can help us stay healthy (Malla et al., 2018). Of all microbes, the most abundant are bacteria, which are ubiquitous in nature and found in every conceivable habitat, from the soil beneath our feet to the depths of the Earth's crust and even in extreme environments like acidic hot springs and areas contaminated with radioactive waste (Bardgett and van der Putten, 2014; Thakur et al., 2022; Rappaport and Oliverio, 2023). Remarkably, bacteria not only coexist with humans and animals but also live within them (Figure 1) (Ursell et al., 2012). These microorganisms showcase incredible diversity in terms of size and shape. Bacterial cells typically range from 0.5 to 5.0 μm in length, with some exceptions such as the giant *Thiomargarita namibiensis*, which can be 50 times larger than most known bacteria (Levin and Angert, 2015; Miller and Murray, 2023). They come in various shapes, including spherical (cocci), rod-shaped

(bacilli), slightly curved rods or comma-shaped (vibrio), spiral-shaped (spirilla), and tightly coiled (spirochaetes). In addition to their physical diversity, bacteria exhibit metabolic versatility (Young, 2006). Their metabolism can be categorized based on their energy source, electron donors, and carbon source. Some, known as phototrophic bacteria, harness energy from light through photosynthesis. Other, chemotrophic bacteria, break down chemical compounds through oxidation, using different electron donors and acceptors in redox reactions (Deusenbery et al., 2021).

These can be further classified as lithotrophs, which utilize inorganic compounds like hydrogen, or organotrophs, which rely on organic compounds. Some bacteria are heterotrophs, obtaining carbon from other organic sources, while others, like blue-green algae, are autotrophs, fixing carbon dioxide for growth (Legendre and Troussellier, 1988; Eiler, 2006). In unique situations, methanotrophic bacteria can utilize methane as both an energy source and a substrate for carbon anabolism. This interconnected and diverse world of bacteria underscores their significance in Earth's ecosystems and their intriguing role in the circle of life (Ahmadi and Lackner, 2024). These bacteria have proven good and bad impacts on human health. While the majority of these are quite helpful a few of these causes' death alarming diseases (Thakur et al., 2019). A fine example of bacterial pathogenesis is Mycobacterium tuberculosis infection (Schmidt and Hensel, 2004). When a person gets infected with *M. tuberculosis* for the first time, the bacteria multiply in the lungs and can spread to nearby lymph nodes and even other parts of the body (Bussi and Gutierrez, 2019). Surprisingly, this initial infection usually doesn't cause any symptoms in adults. However, the person's immune system steps in to control the bacteria's growth and spread (Glickman and Jacobs, 2001). Some bacteria specifically show specificity to the organs and various tissues as in the case of *Neisseria meningitidis* (Rouphael and Stephens, 2012). Other bacteria can be found everywhere in the host body, such as *Staphylococcus aureus* found in most of the tissues from skin to the bloodstream and in various organs (Sender et al., 2016). Despite the strong immune response, the bacteria are rarely eliminated. Instead, *M. tuberculosis* has a remarkable ability to enter a dormant phase, during which the person doesn't show any symptoms of tuberculosis but still carries the bacteria (Kiazyk and Ball, 2017). Also, the degree of infection caused by bacteria can vary from person to person, as it is influenced by the host factors such as host genetics, lifestyle, age, previous infections, nutrition, and environment (Ogunrinola et al., 2020; Hou et al., 2022). The host's susceptibility is also influenced by the surroundings. Environmental pollutants, chemicals, and air pollution all impair the body's ability to fight off bacterial infections (Kraemer et al., 2019). Unlike infectious diseases caused by viruses and parasites, antimicrobial resistance in bacteria is an emerging issue with severe repercussions (Muzyka, 1996).

Overview of bacterial classification

Based on staining

Bacteria are a diverse group of microorganisms and are classified based on various factors, including their structure and function. Staining is a useful method for categorizing bacteria, and it results in two main groups: Gram-positive and Gram-negative bacteria (Liu et al., 2021).

Gram-positive bacteria, like *Staphylococcus aureus, Staphylococcus epidermis, Streptococcus pneumoniae, Clostridium,* and *Bacillus anthracis*, have a single, thick peptidoglycan layer in their cell walls, which makes them "monoderm" (Desvaux et al., 2018; Chateau et al., 2020; Nikolic and Mudgil, 2023). On the other hand, Gram-negative bacteria, such as *E. coli, Klebsiella, Pseudomonas aeruginosa, H. pylori,* and *P. mirabilis*, have a thinner layer of peptidoglycan in their cell walls, which is surrounded by an outer membrane of lipopolysaccharides, making them "diderms" (Moyes et al., 2009).

Gram-positive bacteria appear bluish-purple because their thick peptidoglycan layer retains the crystal violet and iodine, preventing it from washing off. Gram-negative bacteria, however, stain red because their thin peptidoglycan layer cannot hold onto the crystal violet and iodine, and the safranine counterstain takes over (Tripathi and Sapra, 2023). In certain conditions, Gram-positive bacteria can form spores as a survival mechanism when exposed to environmental stress, such as a lack of carbon and nitrogen. These spores help the bacteria endure and potentially cause infections (Becerra et al., 2016). In contrast, Gram-negative bacteria have an additional, permeable outer membrane that requires transport mechanisms across it. This outer membrane contains endotoxins, which contribute to the survival of Gram-negative bacteria. However, Gram-negative bacteria are more dangerous than their Gram-positive counterparts due to their formidable defenses (Hoerr et al., 2012; Ramachandran, 2014). They possess an outer membrane that acts as a barrier, efflux pumps that actively remove antibiotics, and produce enzymes like beta-lactamases that can disarm common drugs (Munita and Arias, 2016). These bacteria can form biofilms, making them resistant to treatment, and can change surface structures to evade the immune system. What's particularly concerning is their propensity for multi-drug resistance, rendering many antibiotics ineffective (Figure 2) (Becerra et al., 2016).

Based on lifestyle

Based on bacterial lifestyle and environmental conditions they can be either intracellular or extracellular. Intracellular bacteria are pathogenic microorganisms capable of establishing a relationship with a susceptible host by multiplying within host cells (Casadevall, 2008). Examples include *Brucella abortus*, *Listeria monocytogenes, Chlamydia trachomatis, Coxiella burnetiid, Mycobacterium tuberculosis*, and *Salmonella enterica* (Drevets et al., 2004; Guo et al., 2023). These pathogens require specific host cells that support their intracellular growth conditions (Ray et al., 2009). They can be categorized as obligate, unable to multiply outside host cells, or facultative, with the ability to multiply both inside and outside cells. Infections caused by intracellular bacteria include brucellosis, listeriosis, tuberculosis, and salmonellosis (Silva, 2012; Jiao et al., 2021). These pathogens use various pathways to enter host cells, such as macrophages, phagocytes, epithelial and endothelial cells, and hepatocyte (Kaufmann, 1993; Thakur et al., 2019). Some may also transmit intercellularly without an

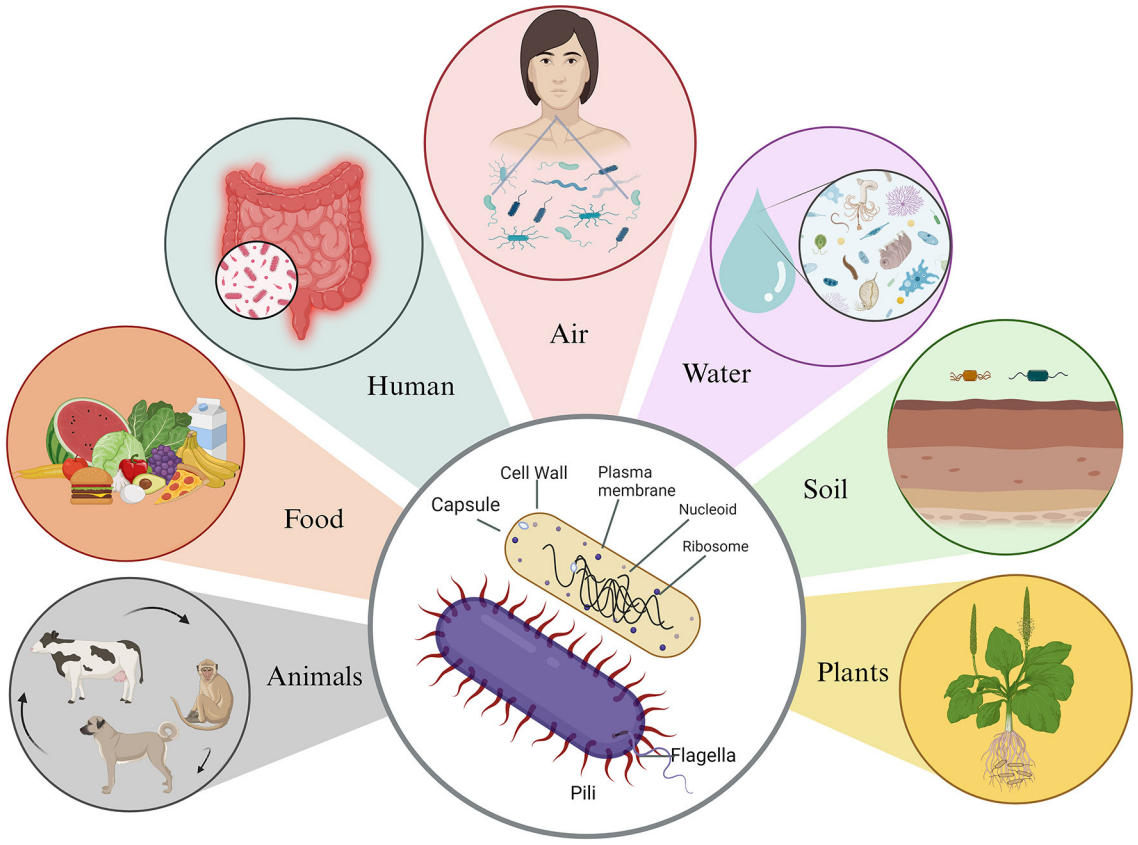

FIGURE 1
Basic structure of bacteria and their associated environmental reservoirs. Bacteria possess a simple body structure without a true nucleus or membrane-bound organelles. Their basic structure comprises the cell wall, which offers structural support and protection, and the plasma membrane, which surrounds the cytoplasm and distinguishes it from the external environment. In addition, some bacteria possess a protective outer layer known as the capsule, which aids them in evading the host's immune system. Bacteria are commonly found in various environments, such as animals, food, humans, air, water, soil, and plants.

extracellular phase. Intracellular bacteria must evade the host's immune response to survive and replicate within mononuclear phagocytes (Kaufmann, 1993; Silva, 2012).

On the other hand, extracellular bacteria, including *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa,* and *Escherichia coli*, exist outside host cells (Belon and Blanc-Potard, 2016). They cause conditions like wound infections, osteomyelitis, scarlet fever, certain types of pneumonia, and urinary tract infections. These pathogens typically multiply in extracellular spaces, such as mucosal surfaces, vascular and lymphatic fluids, and body cavities (Britton and Saunders, 2010; Sansonetti and Puhar, 2010; Mir et al., 2022). Occasionally, they may be found within phagocytes, which are part of the host's defense system. Immune evasion by extracellular bacteria involves mechanisms like humoral immunity and phagocytes, promoting their multiplication outside cells (Pieters, 2001; Schechter et al., 2019). Some extracellular bacteria can behave like intracellular ones in the early stages of infection, including *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumonia, Bacillus anthracis, Escherichia coli, Bordetella pertussis*, and *Helicobacter pylori*, surviving or replicating inside cells *in vitro* and within amoebas (Riffaud et al., 2023). Some extracellular bacteria may not penetrate body tissues but attach to epithelial surfaces and release toxins to cause disease (Schechter et al., 2019).

Bacterial infection and pathogenesis

Pathogenic bacteria are a subset of bacteria which can cause diseases in humans, while most bacteria are harmless or beneficial. The human body hosts thousands of gut flora bacteria (Rolhion and Chassaing, 2016). It encounters various bacteria, including commensals and saprophytes. Defense mechanisms provide innate resistance to microbial invasion. Pathogenic bacteria have evolved mechanisms to overcome this defense and invade the body. Infections usually occur when the body's defense are compromised, due to factors like trauma or underlying diseases. The pathogenicity of bacterial species is determined by their ability to cause disease and symptoms, varying in degree based on their virulence (Shapiro-Ilan et al., 2005). Some bacteria exist in avirulent forms and have little impact on health, but those actively transcribing virulence genes within a host cell can pose significant problems. Virulence factors encompass toxins (such as enterotoxins), surface coats, and

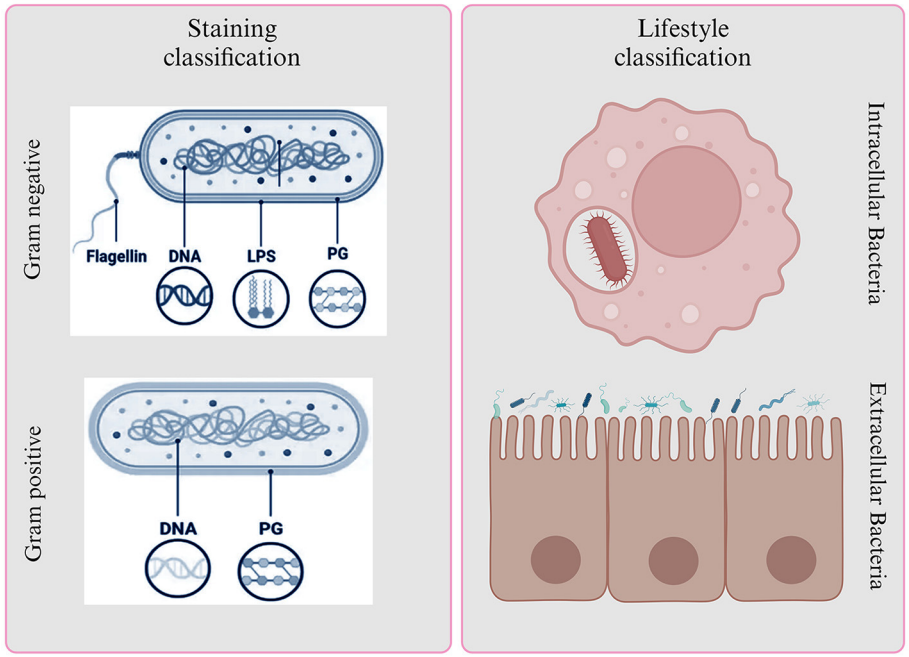

FIGURE 2
Bacterial classification based on Gram staining and lifestyle. It provides insights into their structural characteristics and interactions with host environments.

surface receptors that bind to host cells. As previously mentioned, bacteria play a crucial role in the healthy human body, and any disruption in their balance can lead to disease (Farrell et al., 2021). Bacteria and their host mutually influence each other's activities and functions. Pathogenicity depends on the pathogen's resistance to host defense mechanisms and the host's susceptibility to bacterial virulence factors (Casadevall and Pirofski, 2001). The process of bacterial pathogenesis involves several key steps: contact, colonization, invasion, evasion of host defense, and ultimately infection (Chaffey et al., 2003) (Figure 3).

- *Bacterial exposure* encloses encounters with bacteria via diverse techniques, including contact with surfaces or objects bearing bacterial contamination, the ingestion of food or water harboring bacterial agents, inhalation of airborne bacterial particulates, or direct physical contact with individuals manifesting bacterial infections. Bacterial exposure is a ubiquitous phenomenon in daily existence and manifests the potential for both advantageous and detrimental consequences (Sharma and Gilbert, 2018). On occasions, exposure to commensal or probiotic bacteria can confer immunomodulatory benefits, thereby fortifying the host's immune system or yielding other advantageous effects. Conversely, exposure to pathogenic bacterial strains with virulent attributes can precipitate infectious ailments and maladies (Mazziotta et al., 2023).
- *Bacterial colonization*—Upon encountering a host, certain bacteria exhibit the capability to establish an enduring presence within the host's organism. Bacterial colonization is the process of bacteria initially attaching to a surface or host, then multiplying and forming a stable community (Barron and Young, 2022). This progression is influenced by factors such as the unique traits of the bacteria, the properties of the surface, and environmental conditions (Gollan et al., 2019).
- *Immune system escape*—Bacteria exhibit a remarkable ability to evade immune surveillance a pivotal strategy for their survival within the host environment. This adaptive skillset plays a crucial role following their colonization of the host (Kahn et al., 2002; Finlay and McFadden, 2006). These immune evasion strategies include several sophisticated steps, including inhibiting immune-related signaling pathways, concealing within various host cells, disrupting phagosomes, deactivating reactive oxygen species, and modulating the host's immune response by altering the molecular patterns on their outer surfaces (Van Avondt et al., 2015; Rana et al., 2023). These molecular modifications serve as a camouflage, rendering these bacteria less recognizable to the host's immune receptors. By doing so, bacterial pathogens avoid detection, enabling them to persist and thrive within the host's body (Finlay and McFadden, 2006). The mastery of these intricate camouflage and precision weaponry techniques by bacterial pathogens significantly complicates the development of novel vaccines and innovative treatments. In essence, the bacterial world's ability to navigate and manipulate the host's immune defenses represents a formidable challenge, requiring ingenious approaches to counter their crafty strategies and advance medical interventions (Table 1).
- *Bacterial infection*—Infectious diseases impose an enormous global burden, affecting public health systems and economies worldwide. Although whether the bacteria can cause

symptomatic infection or not depends on its virulence ability as stated above. Majority of the bacteria uses the host for their replication and nutrition purpose without causing much harm (Grant and Hung, 2013; Kiazyk and Ball, 2017). Bacterial diseases can vary depending on the type of organism and the immune system of the host. Numerous pathogenic bacteria display different virulence factors, which can result in a variety of infection signs and symptoms (Fierer et al., 2017). Certain bacteria develop a mutually beneficial relationship with their host and are therefore harmless and possibly even beneficial, but virulent species, such as *Vibrio cholera*, can infect humans and cause serious health problems or even death (Høiby et al., 1986).

Host susceptibility

Host susceptibility to bacterial infections is a complex interplay of various factors. The host's immune system stands at the forefront, with a strong and effective defense crucial in preventing or controlling bacterial invaders (Dropulic and Lederman, 2016). The overall health and immune status of the host are critical in comprehending the host susceptibility. Conditions like malnutrition, chronic illnesses, or immunosuppressive medications can increase susceptibility. Genetic factors also play a role, as some individuals may have genetic variations that influence their vulnerability to specific infections. Age is a significant determinant, with children and the elderly often more susceptible (Burgner et al., 2006). The microbiota inhabiting the host can act as a protective barrier, competing for resources and producing antimicrobial substances. Anatomical and physiological factors are also influential; structures like cilia and mucus in the respiratory tract and the stomach's acidity can deter bacterial growth (Figure 4).

Prior exposures and immunity, acquired through previous infections or vaccinations, can reduce susceptibility to subsequent infections. The environment in which the host lives and works matters, with sanitation, access to clean water, and exposure to contaminated surfaces or vectors affecting the likelihood of infection (Tomalka et al., 2022). Coexisting infections can weaken the host's immune system or create favorable conditions for other bacterial pathogens. The attributes of the bacterial pathogen itself are pivotal. Virulence factors and antibiotic resistance can enhance a pathogen's ability to cause infection (Pan et al., 2020). Understanding these determinants of susceptibility is essential for developing effective strategies for the prevention and treatment of bacterial infections (Fasciana et al., 2019).

Immune evasion

When bacteria target a host cell and choose to invade it for shelter, they employ several evasion strategies to circumvent or neutralize the host's robust immune defenses (Hornef et al., 2002) There are three possibilities arose as the host cell encounters bacteria: (1) the killing/degradation of bacteria by autophagolysosomal fusion, (2) partial digestion of bacteria where the genome of bacteria can persist inside the cell for a longer time and may or may not cause any harm, and (3) where the bacteria successfully evade and survive inside the host cells (Figure 5) (Asrat et al., 2014; Choi et al., 2018; Riebisch et al., 2021). These methods include secreting proteins that can degrade or hinder the host's immune system, modifying the surface of their own membranes, or imitating the actions of host factors. This intricate interplay allows them to establish residence within the host and ensure their safety.

To manage the indigenous colonizing microflora and counteract pathogens, the human body has developed a diverse array of host defense mechanisms. These strategies encompass physical aspects, such as the skin and mucosal linings, mechanical elements like ciliated cells within the respiratory tracts and tight junctions, and biochemical defenses present in bodily fluids like tears and saliva, which contain the antimicrobial enzyme lysozyme (Janeway et al., 2001). These multifaceted defenses are remarkably proficient at preventing the emergence of invasive microbial diseases. Furthermore, the body possesses two adaptable immune defense systems, namely the innate and adaptive immune systems, which can be mobilized as needed (Hilchie et al., 2013).

The innate immune response, the first line of host defense found in all multicellular organisms, predates the acquired immune response. It has evolved to protect the host from a range of toxins and infectious agents like bacteria, fungi, viruses, and parasites. Innate immunity involves preexisting mechanisms like physical barriers, enzymes (e.g., lysozyme), phagocytes, inflammation-related proteins (e.g., complement proteins), antimicrobial peptides, cell receptors (e.g., Toll-like receptors), professional antigen presenting cells, and cells releasing cytokines (Rousset, 2023). However, some bacteria target the protective cells like macrophages (*Listeria monocytogenes*), dendritic cells (*Coxiella burnetii*), and neutrophils (*Chlamydia pneumoniae*). These cells act as a reservoir for many microbes which successfully thrive intracellularly (Gorvel et al., 2014; Mitchell et al., 2016; Kobayashi et al., 2018). It acts rapidly in minutes to hours to prevent infection and initiate the acquired immune response when a new pathogen is encountered. This natural response aims to prevent infection, eliminate invaders, and trigger the adaptive immune response. The innate immune cells recognize bacteria by identifying PAMP-pathogen distinct molecular patterns through certain receptors known as PRRs (pathogen recognition receptors). These receptors can be further classified into various classes, with TLRs (toll-like receptors) being one of the most common. Once these receptors bind with PAMPs, they set off several immune signaling pathways that are necessary for an early infection response and trigger the adaptive immune system to help further control the pathogens. The innate immune response doesn't possess immunological memory and is incapable of recognizing a previously encountered pathogen upon subsequent exposure. Wherein, adaptive immunity is antigen-specific, and takes some time between antigen exposure and the maximal response (Mogensen, 2009).

The adaptive immune response involves identifying specific "foreign" antigens, differentiating them from "self" antigens, initiating pathogen-specific immunologic effectors designed for the precise elimination of pathogens or infected cells, and nurturing immunological memory to enable swift eradication of a particular

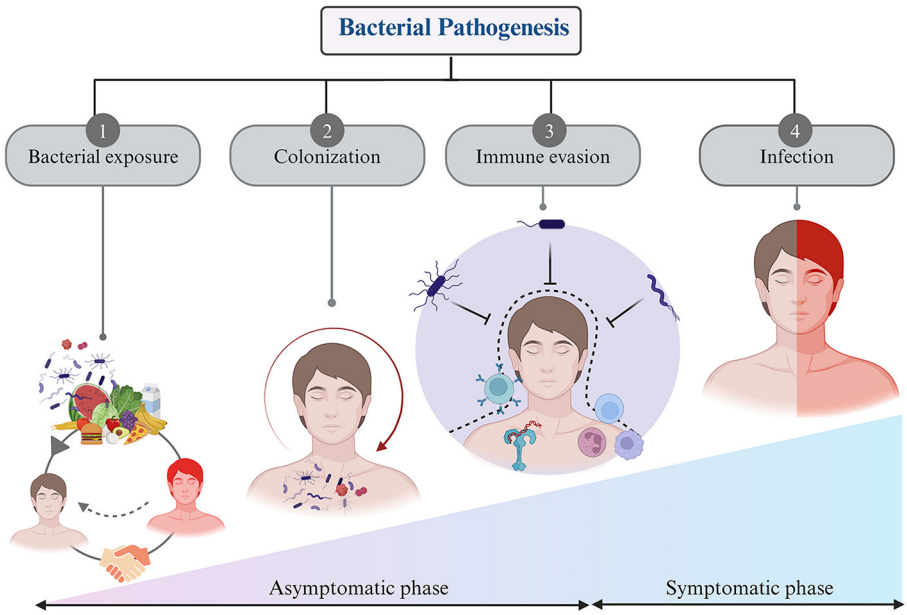

FIGURE 3
Steps of bacterial pathogenesis: (1) Bacterial exposure to host through air, food, infected person, and environment, (2) Colonization of the bacteria inside host within specific organs, tissues, and cells, (3) Evasion of immune response through diverse strategies, and (4) generating negative impact on host by causing infection.

pathogen upon potential reinfections. Adaptive immunity involves a complex network of interactions among B lymphocytes, T lymphocytes, and antigen-presenting cells. B cells perform dual function as they differentiate into plasma cells that produce antibodies along with the production of memory B cells upon activation by foreign invaders. Secondly, they also act as antigen-presenting cell and activates T cells by assisting recognition of a specific antigen, Bacterial pathogens causing infections are transported to macrophages or dendritic cells, influencing the activation of pathogen-specific T cells. On the other hand, pathogens confined to infected tissues may initiate T cell activation through soluble factors, thus impeding a range of host virulence factors (Shepherd and McLaren, 2020). Bacterial virulence factors modulate the immune synapse, aiding the bacteria in evading the immune mechanisms of host cells. This unique aspect of adaptive immunity lies in its ability to establish a memory, enabling a swifter and more efficient immune response upon re-exposure to the antigen (Capitani and Baldari, 2022).

Mechanisms of bacterial immune evasion

i. *Modulation of surface molecules*: Bacteria have several surface features that are critical for immune recognition, including lipid A, flagella, and peptidoglycans. However, bacterial pathogens, particularly gram-negative bacteria, have evolved methods to evade immune detection by altering these molecules. For example, Salmonella uses a two-component sensor to modify lipid A, thereby reduce TLR4 activation. Some bacteria, such as *Porphyromonas gingivalis*, produce diverse lipid A species that selectively moderate inflammatory responses. Peptidoglycan, another essential bacterial molecule, is detected by the intracellular proteins Nod1 and Nod2, triggering an inflammatory response. Pathogens have developed methods to avoid peptidoglycan recognition, and genes involved in its synthesis have been identified as virulence factors. For example, Listeria monocytogenes uses surface-located peptidoglycan hydrolases as virulence factors to exploit Nod2 and promote pathogenesis (Leite Pereira et al., 2020).

ii. *Antigenic variation in bacteria*: Bacterial pathogens, such as *Neisseria* species that cause meningitis and gonorrhea, employ diverse mechanisms for antigenic variation. For instance, *Neisseria*, which causes meningitis and gonorrhea, exhibits antigenic variation through diverse molecular mechanisms. These include multiple copies with independent switches, one expression locus with silent gene copies, and a variable region in the molecules. The constant changes in antigenic molecules, particularly in *Neisseria*, challenge vaccine development and contribute to their ability to survive within the host (Deitsch et al., 2009).

iii. *Subversion of phagocytic cells*: Bacterial pathogens employ diverse strategies to evade killing by phagocytic cells. Internally, pathogens use tactics, such as escape from the phagosome, inhibition of phagosome-lysosome fusion, or survival in phagolysosomes. Some bacteria such as *Shigella* and *Listeria*, secrete lysins to breach vacuolar membranes. Intracellular pathogens manipulate vesicular trafficking. Legionella uses a type IV secretion system, and *Salmonella* employs a Spi-2 type III secretion system and *M. tuberculosis* prevents phagosome acidification. Pathogens modulate inflammatory responses; some activate pathways (e.g., *Shigella* and *Salmonella* induce caspase-1 activation), whereas others dampen inflammation

TABLE 1 Some bacteria-induced tissue-specific diseases and evasion strategies.

S. No	Bacterial species	Bacteria lifestyle	Infection site	Disease	Evasion strategy	References
1.	*Streptococcus pneumoniae*	Intracellular	Upper respiratory tract	Sinusitis, pneumonia, osteomyelitis, septic arthritis	Inhibits neutrophil phagocytosis	Hyams et al., 2010; Subramanian et al., 2019
2.	*Neisseria meningitidis*	Intracellular (facultative)	Nasopharynx	*Neisseria meningitidis*	Dysregulation of nutritional immunity	Coureuil et al., 2019; Mikucki et al., 2022
3.	*Pseudomonas aeruginosa*	Extracellular/ Intracellular	Skin and soft tissue	Endophthalmitis, endocarditis, meningitis, pneumonia	Biofilm generation	Kumar et al., 2022; Kroken et al., 2023
4.	*Mycobacterium tuberculosis*	Intracellular	Lungs	Tuberculosis	Ability to persist in macrophages	Parbhoo et al., 2022; Toniolo et al., 2023
5.	*Listeria monocytogenes*	Intracellular (facultative)	Brain, blood stream	Listeriosis	Escape in cytosol	Osek and Wieczorek, 2022
6.	*Salmonella enterica* Typhi	Intracellular	Gastrointestinal tract	Typhoid fever	Stops fusion lysosome with autophagosome	Wang et al., 2021; Carey et al., 2023
7.	*Rickettsia* spp.	Intracellular	Bones	Rocky Mountain spotted fever, rickettsia pox	Escape into cytosol	Voss et al., 2021
8.	*Chlamydia* spp.	Intracellular	Cervix, urethra, throat and eyes.	Pelvic inflammatory disease (PID)	Degradation of host proteins and deactivation of neutrophils.	Sarkar et al., 2015; Rajeeve et al., 2018
9.	*Anaplasma phagocytophilum*	Intracellular	Neutrophils, granulocytes	Anaplasmosis	Inhibits autophagosome-lysosomal fusion.	Dumler et al., 2005
10.	*Brucella* spp.	Intracellular (facultative)	Liver, heart and central nervous system	Brucellosis	Inhibit fusion with host lysosomal compartment	Jiao et al., 2021

(e.g., Yersinia with YopJ targeting the MAP kinase and NFκB pathways) (Sarantis and Grinstein, 2012).

iv. *Bacterial subversion in innate and adaptive pathways*: Bacterial pathogens employ various strategies to evade the innate immune response. For instance, Yersinia species secrete a virulence factor, inducing immunosuppression through CD-14 and TLR2-dependent signaling, resulting in IL-10 secretion. *Salmonella* adapts to cationic peptides by modifying lipid A, expressing the PgtE protease, and employing a peptide transport locus (sapA-F), all of which are coordinated by the global regulator PhoP/Q. *Salmonella* pathogenicity island 2 (Spi2) shields intracellular Salmonella from reactive nitrogen intermediates, thereby avoiding co-localization with iNOS and NADPH oxidase-mediated killing. Collectively, these mechanisms enable bacteria to circumvent immediate immune responses and establish infection (Baxt et al., 2013).

Bacterial pathogens can also modulate adaptive immune responses. Helicobacter pylori interferes with immune cells by binding to C-type lectin receptors on dendritic cells, resulting in a mixed Th1/Th2 response. The mixed Th1/Th2 response may have implications for the host's ability to combat infection and could contribute to the persistence of the bacteria. Similarly, *N. gonorrhoeae* Opa proteins bind to CD4+ T cells and suppress their activation. *Streptococcal* superantigens alter T-cell distribution and affect disease severity. Mucosal pathogens, such as *Neisseria* and *Haemophilus,* secrete IgA proteases, degrade mucosal antibodies, and impair immune defense.

The challenge of antimicrobial resistance

Bacterial infections pose a significant global burden, with increasing drug resistance exacerbating the problem. Multidrug-resistant bacterial infections are becoming increasingly common, leading to longer hospital stays, increased use of expensive antibiotics, and higher morbidity and mortality rates. Specific examples of bacterial infections with severe consequences include salmonellosis, tuberculosis, and cholera, which collectively cause millions of cases and thousands of deaths each year. *Salmonella enterica serovar Typhimurium* causes ~93.8 million cases of salmonellosis annually, with a death toll of up to 150,000 cases (Galán-Relaño et al., 2023). We have also included specific examples under the subsection of antimicrobial resistance.

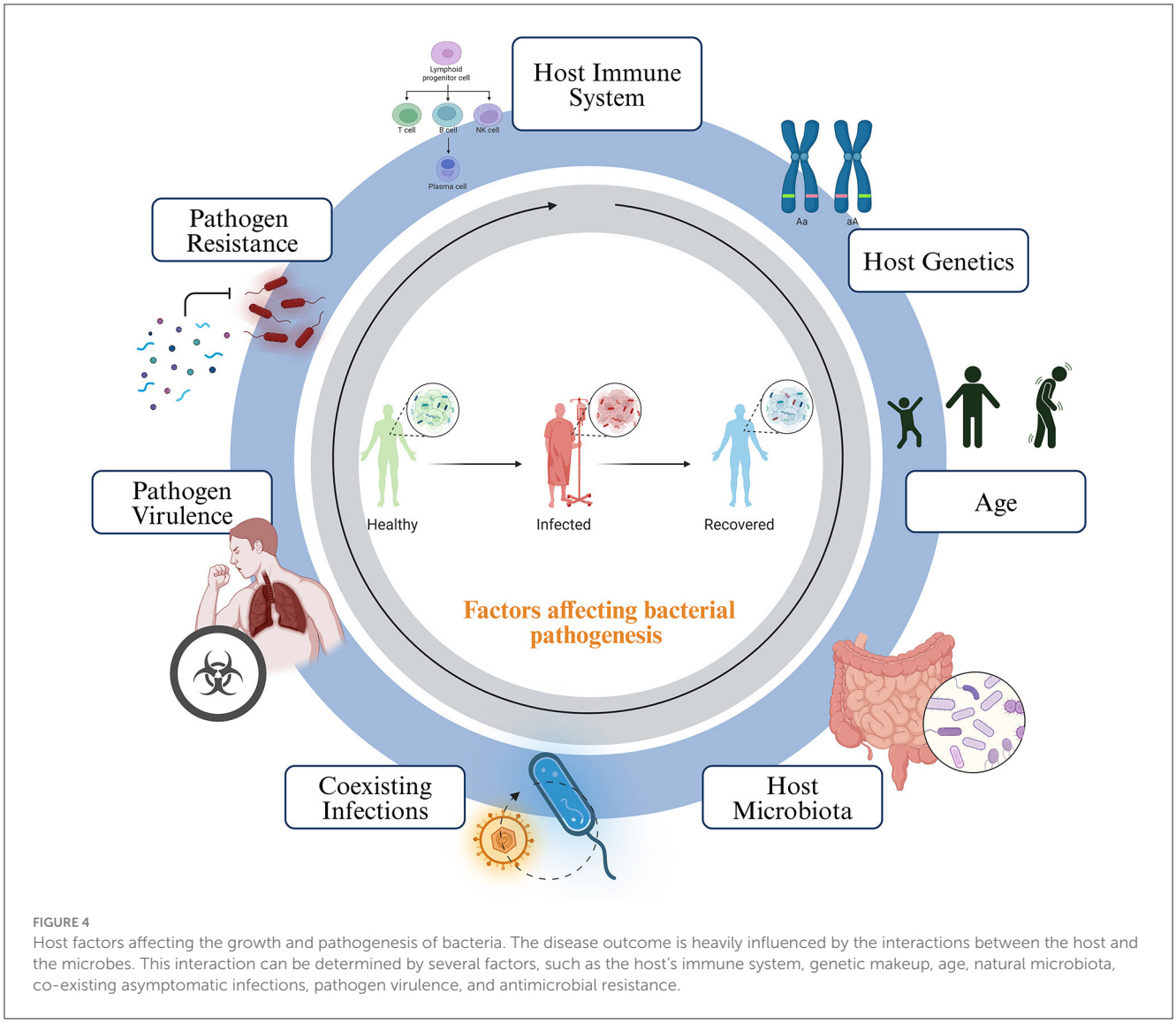

FIGURE 4
Host factors affecting the growth and pathogenesis of bacteria. The disease outcome is heavily influenced by the interactions between the host and the microbes. This interaction can be determined by several factors, such as the host's immune system, genetic makeup, age, natural microbiota, co-existing asymptomatic infections, pathogen virulence, and antimicrobial resistance.

With the introduction of antibiotics, it was initially believed that infection rates would decrease. However, bacteria have demonstrated their ability to outsmart antibiotics, leading to what is commonly known as the antibiotic crisis. As many bacteria have evolved to exhibit antimicrobial resistance, this phenomenon has substantial implications for infectious diseases, ultimately favoring the survival of these resilient bacteria (Michael et al., 2014). Antimicrobial agents target various bacterial mechanisms and processes, including: (1) Inhibition of cell wall synthesis, (2) disruption of protein synthesis, (3) impairment of nucleic acid synthesis, and (4) interference with metabolic pathways. This adaptability of bacteria poses a considerable challenge in the battle against infectious diseases (Reygaert, 2018).

One huge conundrum of antimicrobial resistance is that the use of these drugs leads to increased resistance. Even the use of low or very low concentrations of antimicrobials can lead to selection of high-level resistance in successive bacterial generations, may select for bacteria that are hypermutable strains (increase the mutation rate), may increase the ability to acquire resistance to other antimicrobial agents, and may promote the movement of mobile genetic elements. Bacteria develop resistance through genetic transfer and mutations, often involving plasmids. Stressors like UV radiation can induce mutations, but most are detrimental (Martinez and Baquero, 2000). Resistance mutations mainly impact drug targets, transporters, and modifying enzymes, often reducing the organism's growth rate. Paradoxically, antimicrobial use can boost resistance, even at low levels, promoting hypermutability, cross-resistance, and genetic element transfer.

Antimicrobial resistance mechanisms can be categorized into four main groups:

- *Restricting drug entry*: Bacteria can become antibiotic-resistant by changing their outer membrane's permeability, which serves as a protective barrier. This outer membrane contains protein channels called porins, allowing the entry of substances, including antibiotics. Bacteria can develop resistance by modifying these porins to restrict antibiotic influx. This modification acts as a defense strategy, making it harder for antibiotics to enter the bacterial cell and exert

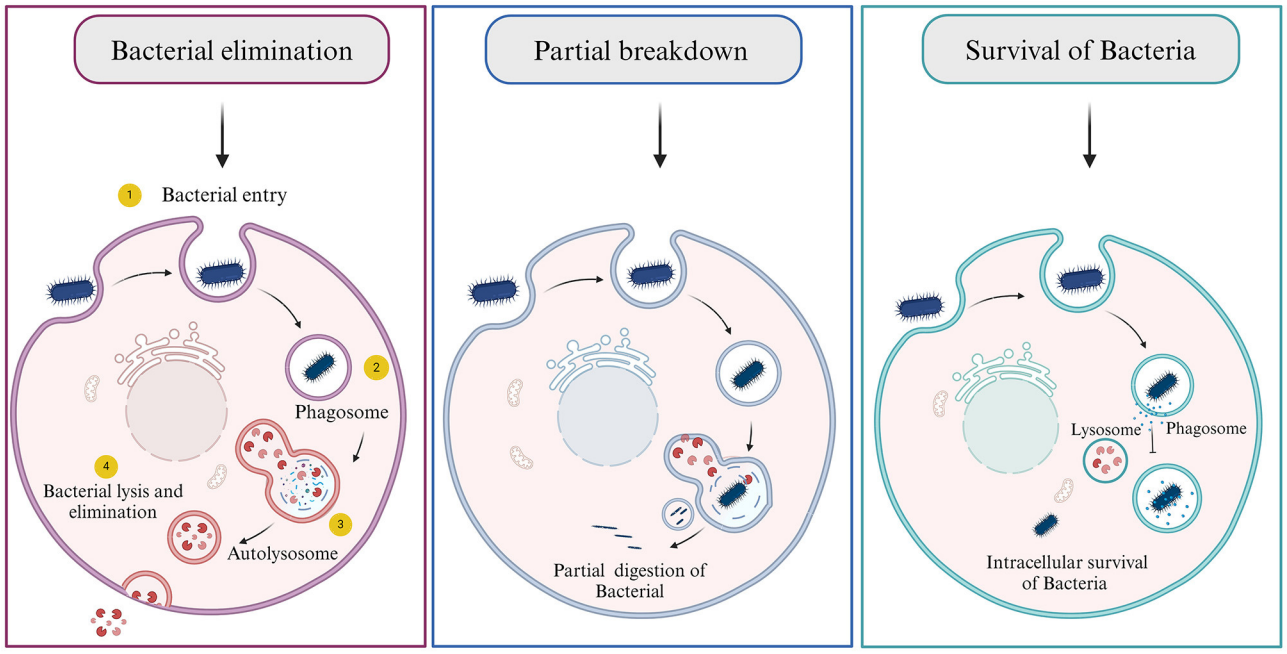

FIGURE 5
Consequences of bacterial infection. (1) Bacterial elimination by host cell, (2) Partial digestion of bacteria, and (3) bacterial escape and survival.

their effects, enabling bacteria to survive in the presence of antibiotics.

- *Altering drug targets*: A successful bacterial strategy against antibiotics involves producing enzymes that either modify the drug or synthesis enzymes that can induce chemical modifications in antimicrobial molecules.
- *Deactivating drugs*: Another strategy involves addition of chemical groups or completely break down the antibiotic, rendering it ineffective in targeting its intended site.
- *Actively expelling drugs*: Bacteria employ efflux pumps as a mechanism to develop antibiotic resistance. These pumps are categorized into various classes, and they can be found in both gram-negative and gram-positive pathogens. Some of these pumps are substrate-specific, designed to expel antibiotics, like the *tet* (tetracycline) determinants for tetracycline and *mef* (macrolides in pneumococci) genes for macrolides in pneumococci. Others have broad substrate specificity and are often associated with multidrug-resistant (MDR) bacteria. Efflux pumps impact a wide range of antimicrobial classes, including inhibitors of protein synthesis, fluoroquinolones, β-lactams, carbapenems, and polymyxins. The genes responsible for encoding these efflux pumps can be in mobile genetic elements (MGEs) or within the bacterial chromosome (Munita and Arias, 2016).

Intrinsic resistance may involve limiting drug entry, deactivating drugs, and drug expulsion, while acquired resistance mechanisms encompass altering drug targets, deactivating drugs, and drug expulsion.

Pathogenic bacteria and their implications in infectious diseases

Various bacterial pathogens are resistant to antimicrobial agents. This increase in antimicrobial resistance among pathogenic bacteria has rendered the treatment of certain common infections, such as pneumonia, exceedingly challenging and, in some cases, nearly impossible. To address this threat to human health, the World Health Organization (WHO) compiled a list of priority pathogens, including *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp., collectively known as ESKAPE. These pathogens alone are responsible for causing >50% of the infection related bacterial deaths globally.

i. *Acinetobacter baumannii* is the most lethal bacterium in bloodstream infections. It exhibits resistance mechanisms such as the production of beta-lactamase, efflux pumps, and enzymatic modification of aminoglycosides and modified porins (Tamma et al., 2022).
ii. *Pseudomonas aeruginosa* demonstrates intrinsic resistance mechanisms, including efflux pump overexpression, reduced outer membrane permeability, and acquired or mutated resistance genes.
iii. *Staphylococcus aureus* rapidly develops antibiotic resistance, including methicillin- and vancomycin-resistant strains.
iv. *Klebsiella pneumoniae* exhibits high antibiotic resistance due to the acquisition of genes encoding enzymes like carbapenemases.

v. *Enterobacter* spp. *and Enterococci* also display various resistance mechanisms. Furthermore, *Escherichia coli (E. coli)* acquires resistance genes through horizontal gene transfer, extended-spectrum β-lactamases, carbapenemases, and plasmid-mediated quinolone resistance genes.

The significant resistance observed in these bacterial pathogens further challenges the treatment of infectious diseases. With the limited advancement for antibiotic therapies and the emergence of multidrug-resistant strains, there is an urgent need to develop new antimicrobial strategies. The threat of antimicrobial resistance not only compromises the ability to combat infections but also presents severe challenges for vulnerable patient populations undergoing medical treatments. As resistance trends persist, it is imperative to explore alternative therapies, invest in drug development, and implement stringent antibiotic stewardship to effectively address the implications of antibiotic-resistant bacterial pathogens (Mulani et al., 2019).

Strategies for combating antimicrobial resistance

- *Drug development*: The increasing prevalence of antimicrobial resistance (AMR) mandates an ongoing quest for novel drug candidates to effectively combat infections. Delving into the mechanisms of AMR not only provides guidance but also functions as a valuable tool in the development for new drugs. Furthermore, the absence of swift and dependable diagnostics has led to inappropriate antibiotic prescriptions in clinical settings, resulting in increased antibiotic exposure and the hastening of resistance development (De Rycker et al., 2018). To tackle the challenge of drug development in the context of AMR, one potential solution is the exploration of diverse genetic interactions to create innovative drug combinations against antibiotic-resistant bacteria. Recognizing that chemical-genetic signatures are unique to each species offers an opportunity to expedite the creation of specific drug combinations. These precisely targeted approaches can amplify the efficacy of antibiotics and enhance AMR management (Silver, 2022).
- *Vaccine development*: AMR is a severe global health issue. Developing vaccines targeting resistant pathogens is a promising solution. To combat antimicrobial resistance, vaccines are a crucial weapon. The pathogens that cause infections are directly inhibited from spreading by a vaccine. Reducing this spread lowers the overall number of infections and lowers the likelihood that a pathogen will evolve into a form that is resistant to drugs. Research into pathogenesis and immune responses informs vaccine design. New adjuvants can enhance protein-based vaccine effectiveness. Simple and cost-effective older vaccine technologies, like live attenuated and inactivated vaccines, are viable. Presently, cost-effectiveness analyses often overlook AMR's impact on vaccine value. To combat this threat to global health, increasing vaccination coverage, enhancing efficacy, and accounting for AMR effects in evaluations are essential steps (Micoli et al., 2021).

Bacterial pathogenicity and its impact on public health

The presence and pathogenicity of bacteria in healthcare units pose significant challenges to public health, affecting both communities and healthcare systems in various ways. Bacterial infections have a large impact on public health and can be transmitted through physical contact, air, water, food, or living vectors. The impact of bacterial pathogenicity on public health includes the transmission of pathogenic bacteria from healthcare setups which contributes to increased morbidity and mortality amongst the immune-compromised patients (Peacock and Newton, 2008). The treatment of these infections requires extended hospital stays, specialized medications, and intensive care, contributing to elevated healthcare costs. Medical infrastructures like hospitals, clinics, and primary health centers serve as potential reservoirs for broader community transmission.

Preventive measures, such as water treatment, immunization, personal hygiene, infection prevention, and control in healthcare settings, have a dramatic impact on reducing morbidity and mortality. Bacteria, such as *Mycobacterium tuberculosis* contribute to the global burden of diseases, straining healthcare resources. *Helicobacter pylori*'s impact on the stomach is linked to various gastrointestinal diseases, affecting community health. Gram-positive bacteria (e.g., *Staphylococcus aureus*, and *Streptococcus pneumoniae*) cause widespread illnesses, impacting both individuals and healthcare infrastructure. *Pseudomonas aeruginosa* infections pose challenges in healthcare settings, demanding extensive resources for patient care (GBD 2019 Antimicrobial Resistance Collaborators, 2022).

Implications for healthcare systems

Prioritizing epidemiologic surveillance allows for effective monitoring and control of infections, aiding early detection and outbreak management. Healthcare workers face increased occupational health risks, emphasizing the need for stringent protocols including regular cleaning and safety guidelines. Establishing and adhering to these measures mitigates the impact of bacterial contamination on both patients and healthcare personnel. Identifying the prevalent bacteria in healthcare units would enable the prioritization of public health actions, including tailored protocols and guidelines. Community education through healthcare systems minimizes the spread of infections beyond healthcare units and promotes responsible health practices.

Limitations and potential shortcomings

Diverse arrays of bacteria and their distinct immune evasion and survival strategies present a formidable challenge for comprehensive coverage in a single review. Each bacterial species exhibits unique methods for evading the host immune system, making comprehensive coverage in a single review a formidable task. Temporal considerations and the dynamic

nature of bacterial pathogens pose complexities. The increasing prevalence of antimicrobial resistance (AMR) further adds on a layer of complexity that influences our understanding of well-known bacterial pathogens and their resistance patterns to specific drugs. Exploring bacterial pathogens at the molecular level poses technological challenges, whereas the intricate environmental conditions crucial for bacterial behavior are challenging to control. Moreover, the variability in host response to bacterial infections, driven by genetic, immune, and health dissimilarity, further complicates the understanding of bacterial pathogenesis.

Conclusion

Bacterial pathogens exhibit remarkable diversity in colonizing various niches within the human body and employing sophisticated strategies for survival and replication. The severity of infection depends on multiple factors, of which immune evasion is of paramount importance. These evasion mechanisms include inhibition of immune signaling pathways, internalize within host cells, disrupting phagosomes, deactivating reactive oxygen species, and modulating the host immune response. Host susceptibility, which is also influenced by genetic polymorphisms, introduces significant variability in the type and intensity of responses to the encountered pathogens.

The rapid emergence of antibiotic resistance poses a formidable challenge. The inherent ability of bacteria to resist antibiotic exposure exacerbates genetic changes caused by inappropriate antibiotic practices. The overuse of antibiotics not only eliminates susceptible bacteria but also facilitates the proliferation of drug-resistant strains. Factors like insufficient sanitation, poor infection control practices, and the widespread application of antibiotics in animal husbandry add to the alarming surge of antimicrobial resistance. Resolving this critical problem necessitates a comprehensive strategy that includes judicious antibiotic usage, better sanitation practices, and increased awareness of the effects of antimicrobial resistance on public health.

While this review provides an overview of bacterial pathogenesis and the emerging antimicrobial resistance crisis, there is a pressing need for further research and in-depth knowledge to enhance our understanding of bacterial pathogens. Future research on bacterial pathogenesis and infections should focus on investigating new antibiotic resistance mechanisms, particularly in bacteria that are evolving, like *E. coli*. Exploring horizontal gene transfer dynamics and understanding zoonotic transmission can help prevent the spread of resistance genes. Embracing a wholistic "One Health" approach that integrates human, animal, and environmental health is crucial for addressing the complexity of bacterial infections. Investing in the development of new drugs and antibiotics, comprehending host-pathogen interactions, and building rapid diagnostic tools are crucial aspects of effective intervention strategies.

Author contributions

RP: Conceptualization, Funding acquisition, Project administration, Supervision, Visualization, Writing – review & editing. JS: Data curation, Investigation, Visualization, Writing – original draft. SS: Investigation, Resources, Writing – original draft.

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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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Toxoplasma gondii Me49 and NED strains arrest host cell cycle progression and alter chromosome segregation in a strain-independent manner

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Toxoplasma gondii is an obligate intracellular parasite that modulates a broad range of host cell functions to guarantee its intracellular development and replication. *T. gondii* includes three classical clonal lineages exhibiting different degrees of virulence. Regarding the genetic diversity of *T. gondii* circulating in Europe, type II strains and, to a lesser extent, type III strains are the dominant populations, both in humans and animals. Infections with the type I strain led to widespread parasite dissemination and death in mice, while type III is considered avirulent. Previously, we demonstrated that primary endothelial cells infected with the *T. gondii* RH strain (haplotype I) were arrested in the G2/M-phase transition, triggering cytokinesis failure and chromosome missegregation. Since *T. gondii* haplotypes differ in their virulence, we here studied whether *T. gondii*-driven host cell cycle perturbation is strain-dependent. Primary endothelial cells were infected with *T. gondii* Me49 (type II strain) or NED (type III strain), and their growth kinetics were compared up to cell lysis (6–30 h p. i.). In this study, only slight differences in the onset of full proliferation were observed, and developmental data in principle matched those of the RH strain. FACS-based DNA quantification to estimate cell proportions experiencing different cell cycle phases (G0/1-, S-, and G2/M-phase) revealed that Me49 and NED strains both arrested the host cell cycle in the S-phase. Cyclins A2 and B1 as key molecules of S- and M-phase were not changed by Me49 infection, while NED infection induced cyclin B1 upregulation. To analyze parasite-driven alterations during mitosis, we demonstrated that both Me49 and NED infections led to impaired host cellular chromosome segregation and irregular centriole overduplication. Moreover, in line with the RH strain, both strains boosted the proportion of binucleated cells within infected endothelial cell layers, thereby indicating enhanced cytokinesis failure. Taken together, we demonstrate that all parasite-driven host cell cycle arrest, chromosome missegregation, and binucleated phenotypes are *T. gondii*-specific but strain independent.

KEYWORDS

***Toxoplasma gondii*, haplotypes, Me49 strain, NED strain, cell cycle arrest, cell cycle dysregulation**

1 Introduction

Toxoplasma gondii is a major zoonotic obligate intracellular apicomplexan parasite and the etiological agent of toxoplasmosis, which may cause harmful effects mainly in pregnant and immunocompromised hosts. *T. gondii* modulates a broad range of host cell functions to guarantee its intracellular development and replication (Velásquez et al., 2019; Fernández-Escobar et al., 2022). As a polyxenous and cosmopolitan zoonotic parasite, *T. gondii* can infect all warm-blooded animals as intermediate hosts (humans, domestic and wild mammals, and birds) and domestic and wild felines as definitive hosts (Gubbels et al., 2008; White and Suvorova, 2018). Hence, its life cycle is a complex transmission process, and detailed molecular knowledge is of importance not only for public health but also for the livestock industry and wildlife management programs (Calero-Bernal et al., 2022).

Worldwide studies have shown that *T. gondii* possesses significant genetic and phenotypic diversity; at present, three main lineages (types I–III) are described, which vary in virulence and mortality for laboratory mice (Fernández-Escobar et al., 2021). Concerning the genetic diversity of *T. gondii* strains circulating in Europe, type II strains and, to a lesser extent, type III strains are the dominant populations, both in humans and animals (Khan et al., 2007; Lorenzi et al., 2016; Fernández-Escobar et al., 2022). The *T. gondii* type I strain is classified as highly virulent, leading to widespread parasite dissemination and lethal infection in mice (100% cumulative mortality). In contrast, mouse mortality and tachyzoite dissemination induced by type II or III strains are considerably lower (30%), with type III strains generally being considered avirulent for mice (Sibley and Boothroyd, 1992; Su et al., 2002; Dardé et al., 2014; Calero-Bernal et al., 2022). The main representatives for each lineage are *T. gondii* RH and GT1 for haplotype I, PRU and Me49 for haplotype II, and CEP and NED for haplotype III (Wu et al., 2022). However, the most studied strain in *in vitro* research is the *T. gondii* RH strain, while the NED strain is commonly used in murine *in vivo* models (Croken et al., 2014; Wang and Sibley, 2020).

It is well known that apicomplexan coccidian parasites extensively modulate their host cells to guarantee successful intracellular development and proliferation. As such, these parasites were reported to affect numerous host cellular processes, such as apoptosis, autophagy, cytoskeleton, metabolism, immune reactions, and cell cycle (Alberts et al., 2007; Dardé et al., 2014; Velásquez et al., 2019). Referring to the latter, the *T. gondii* RH strain arrested the host cell cycle in S-phase or at G2/M-phase transition, which was accompanied by a binucleated host cell phenotype and cytokinesis failure (Molestina et al., 2008; Velásquez et al., 2019). Moreover, RH strain-infected primary endothelial host cells showed supernumerary centrosomes in mitotic spindles, a displacement of single chromosomes from the equatorial plane, and dramatic chromosome missegregation errors (Velásquez et al., 2019). In mammals, the cell cycle is tightly regulated by several cell cycle-dependent cyclins and cyclin-dependent protein kinases (CDKs) that control cell cycle progression from G0/1 to M-phase (Alberts et al., 2007). During cell cycle, the formation of centrioles, the mitotic spindle, and the arrangement of chromosomes are also highly controlled to guarantee the correct genetic information to be inherited by daughter cells (Miettinen et al., 2019). Even tiny errors in these cellular processes might result in genome/chromosome instability or even cell death (Alberts et al., 2007). Former data on *T. gondii* RH infections stated cell type-dependent variations since human foreskin fibroblasts (HFFs) were arrested at the G2-to-M-boundary, while human trophoblast cells, human dermal fibroblasts, and L6 rat myoblasts showed stasis in G2-phase (Brunet et al., 2008; Molestina et al., 2008; Kim et al., 2016). In these studies G2-phase arrest was linked to cyclin B1 downregulation, while other G2/M-phase checkpoint-related molecules, such as p53, p21, and CDK1, were not changed in expression (Brunet et al., 2008; Velásquez et al., 2019). We recently demonstrated that *T. gondii* RH strain-infected primary endothelial cells experienced aberrant mitosis with supernumerary centrosome (and centriole) formation, resulting in impaired cytokinesis (Velásquez et al., 2019). Given that these data referred to haplotype I-driven host cell modulation, we aimed to determine whether cell cycle-related *T. gondii*-driven effects were haplotype/strain-dependent. For direct comparison and to avoid cell type-driven effects, *in vitro* infections with *T. gondii* haplotypes II and III (i.e., Me49 and NED strain, respectively) were performed in the same bovine primary endothelial host cell (BUVEC) as previously used for RH strain.

2 Materials and methods

2.1 Primary bovine umbilical vein endothelial cell isolation and culture

Primary bovine umbilical vein endothelial cells BUVEC were isolated from umbilical veins obtained from calves born by *section caesarea* at the Justus Liebig University in Giessen, Germany. Umbilical cords were maintained at 4°C in sterile 0.9% HBSS-HEPES buffer (pH 7.4; Gibco, Grand Island, NY, United States) supplemented with 1% penicillin (500 U/mL; Sigma, St. Louis, MO, United States) and streptomycin (500 µg/mL; Sigma) for a maximum of 12 h before use. Isolation of endothelial cells was performed by using 0.025% collagenase type II (Worthington Biochemical Corporation) suspended in Pucks solution (Gibco) and infused into the lumen of ligated umbilical veins for 20 min at 37°C in a 5% CO_2 atmosphere. After gently massaging umbilical veins, the cell suspension was collected in medium and supplemented with 1 mL of fetal calf serum (FCS; Gibco) to inactivate collagenase. After two washes (350 x *g*, 12 min, RT), cells were resuspended in complete endothelial cell growth medium (ECGM, PromoCell, supplemented with 10% FCS), plated in 25 cm^2 tissue plastic culture flasks (Greiner), and incubated at 37°C and 5% CO_2 atmosphere. BUVEC monolayers were cultured in modified ECGM medium [ECGM, diluted at 30% in M199 medium, supplemented with 5% FCS (Greiner) and 1% penicillin and streptomycin] with medium changes every 2–3 days. All biological isolates were used for *in vitro* experiments at a maximum of four passages, as previously described in Velásquez et al. (2019). Experiments on bovine primary endothelial cells and parasites were performed following the permission of the Institute of Parasitology to work with biological agents up to risk class 3** [allowance according to §16 BiostoffVO, Az. GI 000056837, approved by the regional commission of Giessen (Regierungspräsidium Gießen)], the Institutional Ethics Commission of Justus Liebig University of Giessen (Germany), and under the current European Animal Welfare Legislation: ART13TFEU.

2.2 Parasite maintenance

Tachyzoites of *T. gondii* Me49 and NED strains were maintained by serial passages in MARC-145 (African green monkey kidney epithelial cells) using DMEM medium (D6429, Sigma) supplemented with 5% FCS, 1% penicillin (500 U/mL; Sigma St. Louis, MO, United States), and streptomycin (500 µg/mL; Sigma). The number of passages for MARC-145 cells and *T. gondii* tachyzoites was controlled to compare our previous results on RH strains with those described here (Velásquez et al., 2019). *T. gondii* tachyzoites were obtained by monolayer scraping and centrifugation (400 x *g*, 1 min) to remove cell debris. A second centrifugation step was performed to sediment the parasites at 800 x *g* for 12 min. Tachyzoites were counted in a Neubauer chamber, suspended in a modified ECGM medium, and used for BUVEC infections.

2.3 Kinetics of *Toxoplasma gondii* infections

Confluent BUVEC layers ($n = 3$) were infected with *T. gondii* Me49 or NED tachyzoites (MOI 1:2) and incubated at 37°C in a 5% CO_2 atmosphere. By counting the number of tachyzoites per parasitophorous vacuole (PV) every 6 h up to 30 h p. i., parasite infection kinetics in the same primary endothelial cell isolates were analyzed to estimate Me49- or NED-specific division cycles. Therefore, cells were fixed at each time point with 4% paraformaldehyde for 15 min at room temperature (RT) and then washed three times with 1X PBS buffer (137 mM sodium chloride, 2.7 mM potassium chloride, and 12 mM total phosphate in the form of hydrogen phosphate and dihydrogen phosphate). All fixed cells were stored at 4°C until further use. To assess parasite development in endothelial host cells, cell nuclei, and tachyzoites were labeled with 4′,6′-diamidin-2-phenyllindol (DAPI) and a specific *T. gondii* antibody, respectively (Table 1), allowing us to count the total number of tachyzoites/PV formed during infection.

2.4 Immunofluorescence assays

Three BUVEC isolates were seeded in 12-well plates with coverslips precoated with fibronectin (1:400, Sigma-Aldrich, F1141-2MG) and infected either with *T. gondii* Me49 or NED tachyzoites at sub-confluency (MOI 1:2). At 24 h p. i., all samples were fixed in 4% paraformaldehyde (15 min, RT) and washed three times in sterile PBS. The samples were incubated in a blocking/permeabilization solution (PBS with 3% BSA and 0.3% Triton X-100) for 1 h at RT. Thereafter, they were incubated in primary antibody solutions (Table 1) at 4°C in a humidified chamber overnight. The samples were then washed three times with 1X PBS and incubated in secondary antibody solutions (Table 1) for 30 min at RT and darkness. Host cell nuclei were labeled with DAPI present in the mounting medium solution (Fluoromount G-DAPI, Thermo Fisher, cat. Number 495952). The samples were analyzed with ReScan Confocal instrumentation (RCM 1.1 Visible, Confocal.nl) combined with a Nikon Eclipse Ti2-A inverted microscope.

2.5 Protein extraction

Six BUVEC isolates were infected with either *T. gondii* Me49 or NED tachyzoites (MOI 1:2). At 24 h p. i., cells were washed with 1X PBS buffer, detached from the plate using trypsin/EDTA solution [0.25% (w/v) Trypsin; 0.53 mM EDTA, 37°C, 5 min], and pelleted (400 *x g*, 5 min). The cell pellet was washed with 1X PBS buffer and resuspended in RIPA buffer (50 mM Tris–HCl, pH 7.4; 1% NP-40; 0.5% Na-deoxycholate; 0.1% SDS; 150 mM NaCl; 2 mM EDTA; 50 mM NaF; all Roth) supplemented with a protease inhibitor cocktail (Sigma-Aldrich), 1 mM sodium orthovanadate tyrosine phosphatase inhibitor (Abcam, ab120386), and 1 mM phenylmethylsulphonyl fluoride, a serine protease inhibitor (Abcam, ab141032). Protein extracts were sonicated for five cycles of 20 s sonication and 20 s resting and then centrifuged (10,000 x *g*, 10 min, 4°C) to sediment intact cells, membranes, and nuclei. The supernatants were analyzed for protein content via BCA protein assay (Pierce BCA Protein Assay Kit, Thermo Scientific, cat. Number 23225) following the

TABLE 1 Primary and secondary antibodies used in the current study.

Antigen	Company	Cat. number	Origin/reactivity	Dilution
Primary antibodies				
T. gondii	Thermo Fisher	PA1-7256	Goat	1:100
γ-Tubulin	Abcam	Ab1795030	Rabbit	1:100
Vinculin	Santa Cruz	sc-73614	Mouse	1:1000
Cyclin A2	Abcam	Ab38	Mouse	1:1000
Cyclin B1	Abcam	Ab32053	Rabbit	1:3000

Antigen/Conjugate	Company	Cat. number	Host/target	Dilution
Secondary antibodies				
Alexa Fluor 594	Thermo Fisher	A-21468	Goat	1:500
Alexa Fluor 647	Thermo Fisher	A-21244	Rabbit	1:500
Goat anti-mouse IgG Peroxidase conjugated	Pierce	31430	Goat/mouse	1:40,000
Goat anti-rabbit IgG Peroxidase conjugated	Pierce	31460	Goat/rabbit	1:40,000

manufacturer's instructions. Sample analysis was performed on a Varioskan plate reader, measuring the absorbance at 562 nm.

2.6 SDS-PAGE and immunoblotting

Protein extracts were diluted in loading buffer with 6 M urea (10% SDS, 12.5% 2-mercaptoethanol, 25% glycerol, 150 mM Tris–HCl pH 6.8) and boiled at 95°C for 5 min. Samples (40 µg protein/slot) were loaded on 12% polyacrylamide gels and subjected to SDS-PAGE electrophoresis (100 V; approx. 1.5 h; Bio-Rad). Proteins were transferred to PVDF membranes (Millipore) (300 mA, 2 h) in a wet-tank transfer system and then blocked for 1 h at RT [3% BSA in TBS buffer (50 mM Tris–HCl, 150 mM NaCl; pH 7.6)]. Afterwards, proteins on membranes were incubated in primary antibodies (4°C, overnight) directed against cyclin A2, cyclin B1, and vinculin (Table 1) and diluted in blocking solution (TBS buffer, 0.1% Tween-20, 3% BSA). Vinculin detection was used as a loading control for sample normalization. After primary antibody probing, membranes were washed three times with TBS-Tween (0.1%) and incubated in secondary antibody solutions (Table 1) for 30 min at RT. After three washings (TBS-Tween 0.1%), protein detection was performed using a chemiluminescence detection system (ECL Prime, Amersham). Images were taken using the INTAS Science Imaging Instrument and the INTAS ChemoStar Imager software. A protein ladder was used to estimate protein sizes (PageRuler Plus Prestained Protein Ladder, Thermo Fisher Scientific). Protein band intensities were analyzed using the Fiji Gel Analyzer plugin (Schindelin et al., 2012).

2.7 Flow cytometry-based analysis of cell cycle phases

For flow cytometry (FACS)-based analysis of the cellular DNA content, non-infected and infected host cell layers ($n = 6$) were washed with 1X PBS buffer and detached from the plate by trypsin/EDTA (0.25%) treatments at 37°C for 5 min. Then, cells were pelleted at 400 x *g* for 5 min. The cell pellet was resuspended, washed in 1X PBS buffer, and centrifuged at 400 x *g* for 5 min. Thereafter, cells were fixed with ice-cold absolute ethanol for 3 min at −20°C and pelleted again (400 x *g*, 5 min). Samples were stained with the commercial FxCycle PI/RNAse kit following the manufacturer's instructions (Thermo Fisher, F10797). The samples were analyzed by an Accuri C6 Plus Flow Cytometer analyzer (Becton-Dickinson, Heidelberg, Germany) applying 535/5 nm excitation and emission collected in a 617/20 band-pass. The cells were gated according to their size and granularity. Data analysis was performed via FlowJo LLC software (Ashland, OR).

2.8 Image acquisition and reconstruction

Fluorescence images were acquired with a ReScan Confocal instrumentation (RCM 1.1 Visible, Confocal, The Netherlands) equipped with a fixed 50 µm pinhole and combined with a Nikon Eclipse Ti2-A inverted microscope with a motorized Z-stage (DI1500, Nikon). The RCM unit was connected to a Toptica CLE laser with the following excitation modes: 405/488/561/640 nm. Images were taken via an sCMOS camera (PCO edge) using a CFI Plan Apochromat X60 lambda-immersion oil objective (NA 1.4/0.13; Nikon). The instrument was operated by the NIS-Elements software (version 5.11). Images were acquired via a z-stack optical series with a step size of 0.1 microns to cover all structures of interest. To estimate both the total number of cells and the number of binucleated cells present in one cell layer, all images were first segmented using the Otsu thresholding algorithm. Identical brightness and contrast conditions were applied for each data set within one experiment. The total number of cells was obtained using the Fiji plugin "Analyzes particles" with a size of 10 µm (Schindelin et al., 2012).

2.8.1 Mitosis quantification

The percentage of cells that underwent mitosis was calculated by normalizing it with the total number of cells in each field of view. Mitosis was evaluated from the prophase until the telophase. Cytokinesis was evaluated in terms of the percentage of binucleated cells. The prophase was defined as the chromosome condensation in the nuclear region using DAPI staining of the chromosomes. Metaphase cells were those with all chromosomes in the equatorial line, and anaphase cells were when chromosomes left the central plane to migrate into each mitosis pole. Cells in anaphase with a DAPI-positive signal in between were identified as chromosome bridges. Finally, cells in telophase were counted when chromosomes had completely migrated to each mitosis pole.

2.9 Statistical analysis

The data were expressed as the mean ± SD of independent experiments. For cell number- and FACS-based experiments, one-way analysis of variance (non-parametric ANOVA) with Kruskal-Wallis post-test was performed using GraphPad Prism 9.3.1 software, applying a significance level of 5%. For immunoblot-based analyses, unpaired two-tailed *t*-tests were performed comparing controls vs. infected cells, with a 95% confidence interval. All graphs and statistical analyses were performed using GraphPad Prism 9 software.

3 Results

3.1 *Toxoplasma gondii* Me49 and NED intracellular development show comparable kinetics in primary endothelial cells

The current aim was to evaluate if variable *T. gondii* strains may differentially affect the host cell cycle during tachyzoite intracellular development. Given that diverse *T. gondii* haplotypes bear different virulence in the murine system, which may be linked to varying speed in development and cell lysis, we first analyzed the developmental characteristics of Me49 and NED strains (haplotypes II and III, respectively) in primary BUVEC layers. The rationale to choose a primary bovine endothelial cell type as host cells was: (i) to avoid immortalization-driven effects on cell cycle regulation as commonly reported for permanent tumor-based cell lines, (ii) to be as close as possible to the *in vivo* scenario, and (iii) to perform current studies in exactly the same cell type as reported before for the *T. gondii* RH strain [haplotype I, (Velásquez et al., 2019)] thereby allowing for direct data

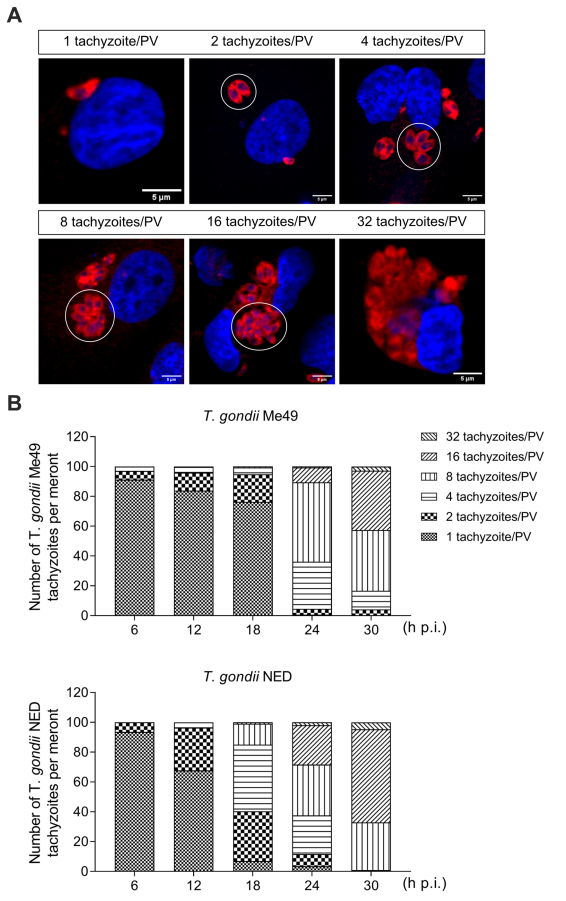

FIGURE 1
Toxoplasma gondii Me49 and NED tachyzoite replication kinetics in primary bovine umbilical vein endothelial cells (BUVEC). BUVEC isolates ($n = 3$) were simultaneously infected at subconfluence with either Me49 or NED tachyzoites (MOI 1:2) and monitored for parasite proliferation by analyzing the numbers of tachyzoites/parasitophorous vacuole (PV, white circle) from 6 to 30 h p. i. **(A)** Representative illustration of different stages of proliferation. Host cell nuclei were stained by DAPI (blue) and tachyzoites by *T. gondii*-specific antibodies (red). **(B)** Estimation of the proliferation status of Me49 and NED strains at 6, 12, 18, 24 and 32 h p. i. The scale bar represents 5 µm.

comparison. Therefore, BUVEC and MARC-145 passages, as well as *T. gondii* tachyzoites, were carefully controlled.

To study the kinetics of parasite development, identical BUVEC isolates were simultaneously infected with tachyzoites of *T. gondii* Me49 and NED strains, and the total intracellular development of each strain was thoroughly assessed by counting the number of tachyzoites per PV every 6 h from 6 to 30 h p. i. (Figure 1). Given that the host cells used in the current study were of primary origin and therefore not immortalized, three biological isolates at a maximum of four passages were used to avoid potential age-dependent changes in cell division times and further to ensure preservation of the endothelial phenotype. All host cells were seeded and infected at the same time, using the same batch of tachyzoites from each strain. A rosette was defined as when the PV contained 32 tachyzoites. All samples were analyzed before 32 h p. i. to avoid cell lysis-driven artifacts.

When analyzing in total 895 and 770 single infected host cells for NED and Me49 strains, respectively, at 24 h p. i., the overall infection rates differed moderately but not substantially (Me49: 81.9 ± 3.1%; NED: 70.1 ± 7.7%). The onset of tachyzoite division revealed equal in both strains at 6 h p. i. 2.5% of infected BUVEC showed two tachyzoites per PV for both strains (Figures 1A,B). However, at 12 and 18 h p. i., the NED strain proceeded slightly faster in development than the ME49 strain, since a higher proportion of PV contained four and eight tachyzoites (ME49: 1.3 and 5.7%, respectively; NED: 2 and 16.9%, respectively) (Figures 1A,B). Nevertheless, towards 30 h p. i., the NED strain caught up in development, thus resulting in a comparable proportion of PV with 32 tachyzoites (ME49: 1.4%; NED: 1%) (Figures 1A,B). Of note, BUVEC lysis started at comparable time points at 33 h p. i., thereby denying any relevant differences in the *in vitro* virulence of these two *T. gondii* strains. Based on these overall findings, it appeared eligible to perform cell cycle-related experiments on both strains at the same time points after parasite infection.

3.2 *Toxoplasma gondii* Me49 and NED tachyzoite infections both induce binucleated host cells and affect centriole formation

It was previously described that *T. gondii* RH strain infection in BUVEC layers resulted in an enhanced proportion of bi/multi-nucleated host cells (thereby indicating cytokinesis failure) and an alteration of mitosis progression by inducing supernumerary centrosome formation and chromosome segregation errors (Molestina et al., 2008; Velásquez et al., 2019). In this study, we intended to study whether these findings also applied to other *T. gondii* haplotypes (i.e., Me49 and NED). Therefore, the number of bi/multi-nucleated host endothelial cells (i.e., with ≥ 2 nuclei per cell) was counted and normalized against the total number of cells present in the field of view. As depicted in Figure 2A, both Me49 and NED infections of BUVEC induced a significantly enhanced proportion of bi/multi-nucleated host cells (Me49: 12.7%; NED: 8.1%), while only 0.7% of non-infected cells revealed the binucleated phenotype. The mitotic rate was also analyzed by estimating the total number of cells in mitosis vs. the total number of cells in the field of view. The results showed that only the NED strain reduced statistically significant mitosis percentages (Figure 2B). To verify whether mitotic failures may be linked to inadequate mitotic spindle and centrosome formation, we additionally stained chromosomes with DAPI (blue) and centrosomes with γ-tubulin (green) (Figure 2C) and analyzed the different phases of mitosis in *T. gondii* Me49- and NED-infected BUVEC. The γ-tubulin staining was used as a centrosome marker to visualize the mitosis spindle localization. As expected, most of the mitotic cells displayed a normal mitosis progression with only two centrosome poles. However, approximately 10% of infected mitotic cells showed an altered chromosome arrangement (Figure 2C, prophase and metaphase). A low number of host cells displayed chromosome bridges between the two poles of the mitotic spindle (Figure 2C, asterisks, and Supplementary Video S2). Interestingly, the centrosomes seem to be composed of more than one spot (Figure 2C, and Supplementary Video S1). To corroborate this observation, we drew a line over the centrosomes in mitotic cells, and we plotted the information in a histogram. The results showed that in most of the cases, the histogram exhibited two brightness dots, suggesting a possible centriole duplication at each centrosome (Figure 2C, histogram plots, and Supplementary Video S1).

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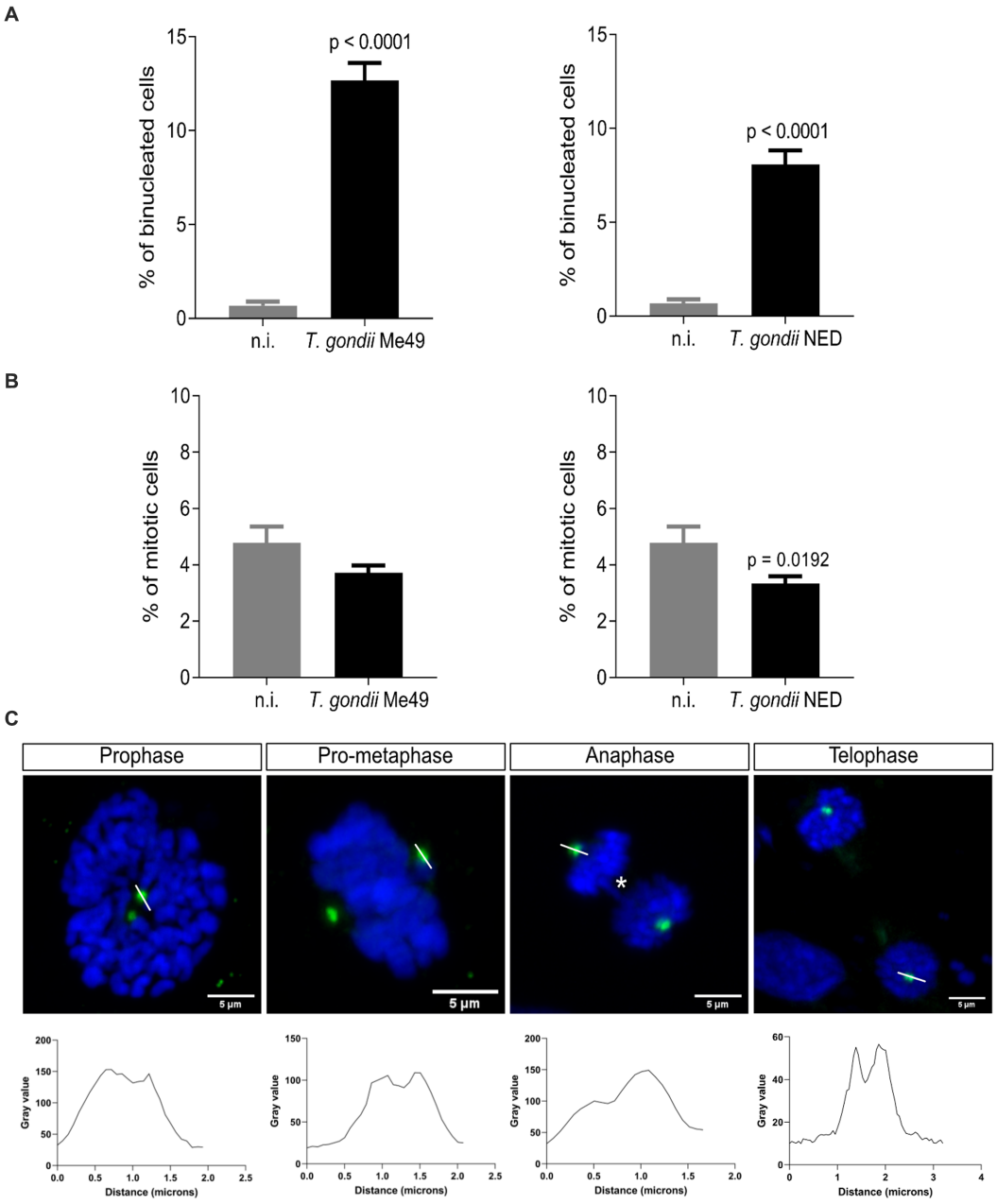

FIGURE 2
Toxoplasma gondii Me49 and NED infections induce a binucleated phenotype and centriole overduplication in infected host cells. Subconfluent BUVEC isolates ($n = 3$) were infected with either Me49 or NED tachyzoites at an MOI 1:2. After 24 h p. i., samples were stained for DNA/chromosomes (DAPI, blue) and centrioles (γ-tubulin, green). **(A)** Percentages of host cells with a binucleated phenotype. **(B)** Mitotic rate (cells undergoing mitosis/total number of cells) of *T. gondii*-infected BUVEC and control cells. **(C)** Exemplary illustration of the main aberrant mitotic structures observed for both Me49 and NED strain infections. Additionally, the intensity of centriole-related signals was assessed and plotted as a graph showing intensity value vs. distance (white line). Yellow arrows: centriole overduplication; asterisks: chromosome bridge. The scale bar represents 5 µm.

3.3 *Toxoplasma gondii* ME49 and NED infections both promote host cell cycle arrest in S-phase

To analyze whether *T. gondii* Me49 or NED strains cause dysregulation of the host cell cycle progression in BUVEC, a FACS-based analysis was performed to estimate total DNA content. The cell population was gated according to their size and granularity, and PI-based DNA staining was used to define distinct categories of cell cycle phases (G0/1, S, and G2/M) according to the DNA amount per cell (Figure 3A). In this classical method, the first DNA peak is assigned to G0/1-phase, the second peak represents cells in G2/M-phase, and the cell population between both peaks corresponds to cells in the S-phase (Figure 3A). Here, *T. gondii* Me49- and NED-infected cells both showed a significant decrease of cells in G0/1-phase when compared to non-infected cells. Simultaneously, the proportion of *T. gondii*-infected cells in the S-phase increased, thereby suggesting a parasite-driven host cell arrest in the S-phase (Figure 3B).

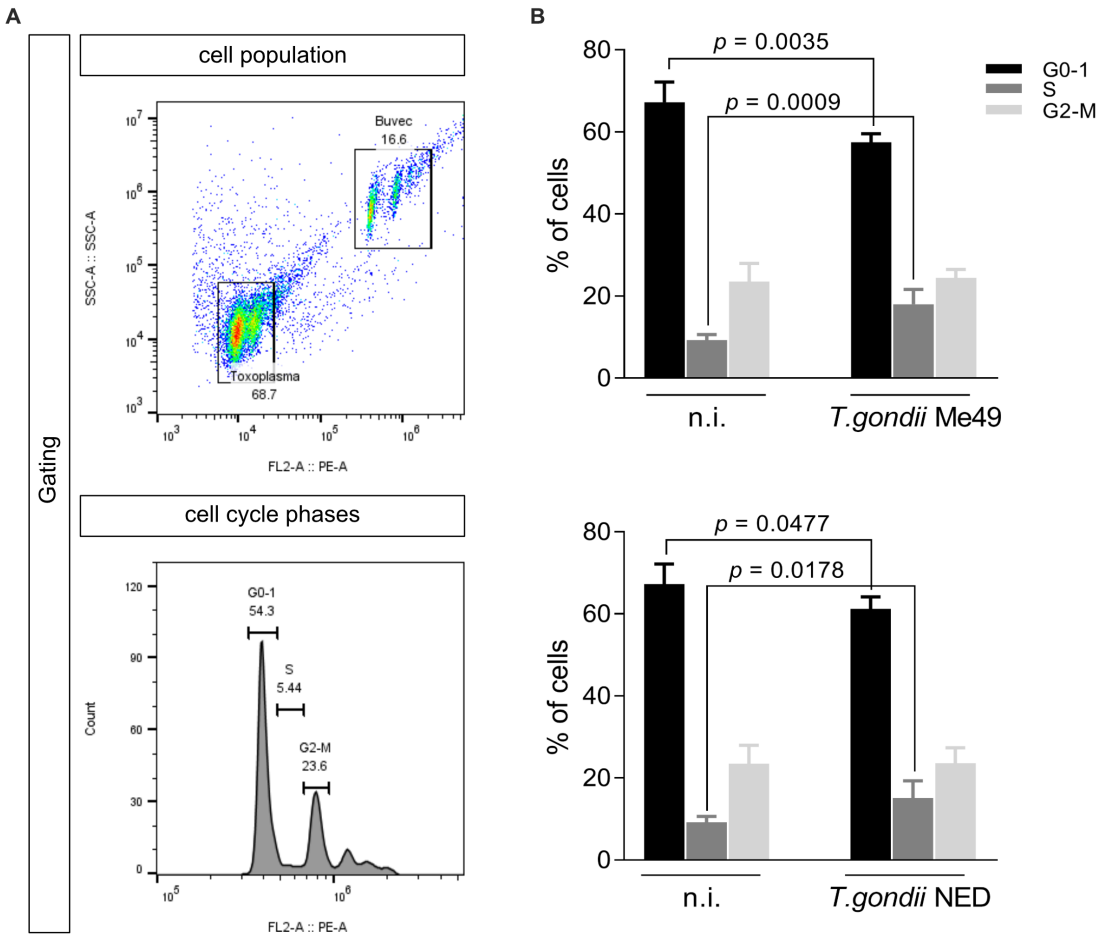

FIGURE 3
Toxoplasma gondii Me49 and NED infections arrest the host cell cycle in S-phase. Subconfluent BUVEC isolates ($n = 6$) were infected with either Me49 or NED tachyzoites at an MOI 1:2 and evaluated for DNA content at 24 h p. i. **(A)** Exemplary flowchart of FACS analysis showing the total number of host cells in G0-1 (one genomic DNA copy), G2- (two genomic copies), and S-phase (DNA synthesis, the cell population in between both phases). The number of cells positive for DNA staining (PI) was graphed as a histogram to obtain the total number of cells in each peak. **(B)** Percentages of Me49- and NED-infected BUVEC and control cells in each cell cycle phase were estimated via FACS-based DNA quantification. Bars represent the median ± SD.

Since cellular DNA content-based analyses do not allow for discrimination between all single phases (such as G0/G1 or G2/M) and based on current data suggesting that *T. gondii*-infected host cells accumulate in the S-phase, we additionally analyzed the expression of cyclins A2 and B1 (Figure 4), which signify key regulatory proteins of S-phase control and of M-phase enter and progression, respectively. Western blotting analyses of six ME49- and NED-infected BUVEC isolates showed that only the NED strain induces cyclin B1 overexpression (Figure 4B). Cyclin A2 expression was not affected by any of the strains (Figures 4A,B).

4 Discussion

In a recent study, we demonstrated that *T. gondii* tachyzoites of haplotype I (RH strain) induced host cell cycle arrest, chromosome missegregation, multipolar spindle, and cytokinesis failure concomitant with an increased percentage of binucleated primary host endothelial cells *in vitro* (Velásquez et al., 2019). To assess eventual strain-driven effects, we compare our previous published data with infections using two other *T. gondii* haplotypes, II and III (Me49 and NED, respectively). We evaluated the host cell cycle control, progression, and mitosis after 24 h h p. i. with *T. gondii* tachyzoites Me49 and NED. To be able to compare our results with previously published data, the same host cell type was used and parasite strain passages were tightly controlled. Furthermore, we followed the same experimental approach used in Velásquez et al. (2019).

To our best knowledge, available data on *T. gondii*-driven host cell cycle modulation currently refer to haplotype I tachyzoites (RH strain) and indicate that this clonal lineage might control host cell cycle progression to ease its intracellular asexual development (Brunet et al., 2008; Molestina et al., 2008; Velásquez et al., 2019; Wong et al., 2020; Pierre-Louis et al., 2022). Since several typical and atypical clonal lineages of *T. gondii* occur worldwide and show variable pathogenicity (Dardé et al., 2014; Miller et al., 2023), we aimed to compare recent RH data (haplotype I) (Velásquez et al., 2019) with haplotypes II and III (Me49 and NED, respectively) by infecting the same host cell type. In our hands, the replication times of the ME49 and NED strains in BUVEC proved comparable to those of the RH strain, allowing us to use this primary cell culture system for comparative approaches. Our

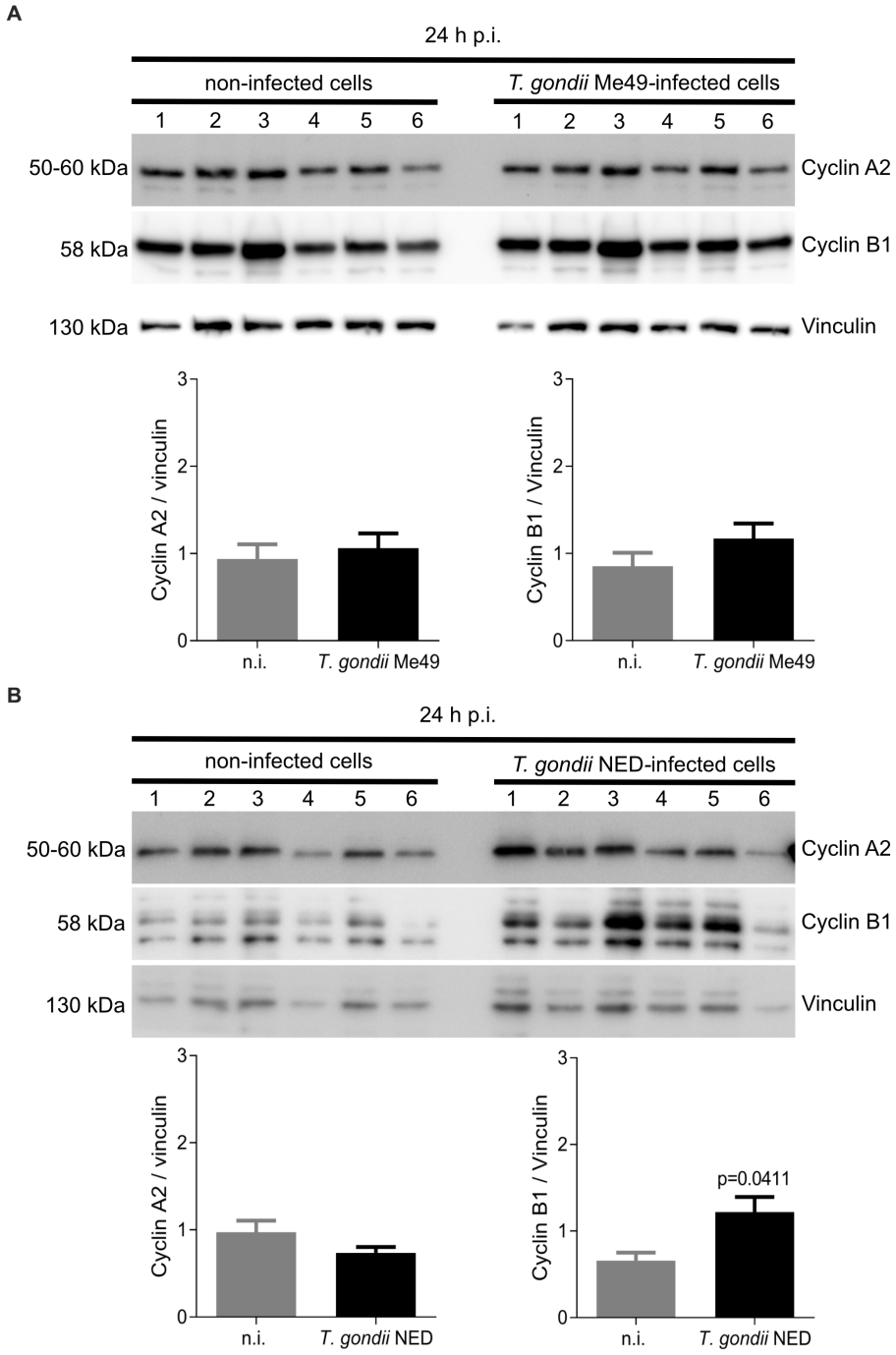

FIGURE 4
Cyclin A2 and cyclin B1 expression in *Toxoplasma gondii* Me49 and NED strain-infected BUVEC. Confluent BUVEC isolates ($n = 6$) were infected with either Me49 **(A)** or NED **(B)** tachyzoites. At 24 h p. i., protein extracts were analyzed by Western blotting to estimate cyclin A2 (indicative for S-phase) and cyclin B1 (mitosis marker) expression. Densities of protein signals were quantified and graphed as a relative ratio to vinculin expression (loading control). Bars represent the median ± SD of six biological replicates.

results showed that the NED strain reduced the mitosis rate and led to an overexpression of cycin B1, in contrast to the Me49 strain. This suggests that the NED strain modulates the host cell cycle throughout the mitosis checkpoint, while ME49 and RH appear to modulate whitin S-phase progression itself. Since neither *T. gondii* RH nor ME49 controls the mitosis checkpoint cyclin, even though both arrest the host cell cycle, *T. gondii* tachyzoite has evolved into several clonal lineages, with the most prevalent ones being types I, II, and III. While all lineages have the potential to infect both humans and animals, types I and II are more prevalent in immunocompromised or pregnant humans than in animals. Interestingly, type III primarily infects animals as opposed to people (Howe and Sibley, 1995). In our findings, the NED strain was the one to have distinct behavior about the cell cycle and mitosis control. Therefore, this slight modification may suggest that the NED

strain evolved into a better-intermediated host adaptation, modifying the type of the host cell cycle and mitosis control. However, further experiments need to be done in order to answer this hypothesis.

Referring to parasite-mediated modulation of cell cycle progression, the current data showed that both NED and ME49 strains induced host cell cycle arrest in S-phase, but they differed in the control, thereby denying any haplotype-dependent reactions. Interestingly, S-phase arrest has been studied by other groups worldwide, suggesting that arrested cells were not able to incorporate new DNA molecules (Pierre-Louis et al., 2022). Given that *T. gondii* arrests the host cell cycle equally in primary, immortalized, and tumor cells, we could suggest that it is a parasite strategy that has been maintained throughout haplotype evolution (Brunet et al., 2008; Molestina et al., 2008; Kim et al., 2016; Velásquez et al., 2019; Pierre-Louis et al., 2022). The mitosis progression was shown to be modulated differently in NED- or ME49-infected cells compared to previous data published on the RH strain since infected cells displayed multipolar spindle formation (Velásquez et al., 2019). In this study, we showed that neither ME49 nor NED strains induce multipolar spindles. However, the centrosome poles seem to have more than one spot of γ-tubulin, suggesting possible centriole duplication. This phenotype was also observed in the RH strain infection, but it always came with a multipolar phenotype. Altogether, this suggests that *T. gondii* modulation of the centriole number can be independent of the strain type, but the multipolar spindle formation may only be induced by the RH strain.

During the process of chromosome segregation and mitotic spindle formation, several specific proteins are needed, such as histones and cohesins, to deliver structural support for chromosomes and to bind sister chromatids to ensure an equal distribution between daughter cells during cell division (Kline-Smith and Walczak, 2004; Mariño-Ramírez et al., 2005; Makrantoni and Marston, 2018). The loss or gain of chromosomes drives chromosomal errors and triggers intracellular pathways that arrest the cell cycle to either repair the damage or eliminate potential aneuploid cells by apoptotic death (Alberts et al., 2007; Levine and Holland, 2018). The same applies to irregular chromosome bridges, which were found here to be induced by *T. gondii* Me49 and NED strains and that have been correlated with DNA damage (Ganem and Pellman, 2012). As described by Pampalona et al. (2016), chromosome bridges are characteristic of tumor cells and are mainly observed in mid-late anaphase, eventually persisting throughout mitosis, but are atypical for the early G1 phase. Since the endothelial host cells used in the current study are of primary and non-tumoral origin and, consequently, should follow physiological cell cycle control, chromosome bridge-related findings should be associated with *T. gondii* infection. However, considering that chromosome bridging is better monitored by live-cell imaging, future experimentation will focus on this method to reliably correlate these findings with *T. gondii*-mediated effects.

In line with recent data on the *T. gondii* RH strain (Velásquez et al., 2019), we demonstrated that ME49 and NED infections of BUVEC also induced an increased percentage of binucleated cells, which directly correlates with enhanced cytokinesis failure. Cytokinesis represents the last pivotal step of cell division. Hence, cytokinesis includes cytoplasmic division finally giving rise to two daughter cells, even when some exceptions have been described in the early embryonic stages of the fruit fly model *Drosophila* (Schejter and Wieschaus, 1993; Kiseleva et al., 2001). In mammals, megakaryocytes (blood platelets), hepatocytes, and heart muscle cells perform nuclear division without cytokinesis, leading to a high proportion of multi-nucleated cells (Nagata et al., 1997; Ahuja et al., 2007; Alberts et al., 2007; Margall-Ducos et al., 2007; Lordier et al., 2008). However, the endothelium of vessels *in vivo* physiologically does not include binucleated phenotypes, therefore evidencing that our findings are directly correlated with *T. gondii* infections. Given that—with the current study—this phenotype is now described for haplotypes I–III *in vitro*, binucleated phenotypes and cytokinesis failure may be considered a specific hallmark of *T. gondii* tachyzoite replication. Whether this phenomenon is indeed not linked to strain virulence seems likely but should be tested *in vivo* for further clarification.

Taking all the data together, we can suggest that host cell cycle modulation, chromosome segregation, and cytokinesis failure are intrinsic mechanisms of *T. gondii* tachyzoite infection and are independent of the parasite haplotype or virulence.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The experiments on bovine primary endothelial cells and parasites were performed following the permission of the Institute of Parasitology to work with biological agents up to risk class 3** [allowance according to §16 BiostoffVO, Az. GI 000056837, approved by the regional commission of Giessen (Regierungspräsidium Gießen)], Institutional Ethics Commission of Justus Liebig University of Giessen (Germany), and under the current European Animal Welfare Legislation: ART13TFEU. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LR-B: Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. CH: Funding acquisition, Resources, Writing – review & editing. AT: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing. ZV: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1336267/full#supplementary-material

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Peptide selection via phage display to inhibit *Leishmania*-macrophage interactions

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Introduction: Leishmaniasis comprises a complex group of diseases caused by protozoan parasites from the *Leishmania* genus, presenting a significant threat to human health. Infection starts by the release into the skin of metacyclic promastigote (MP) form of the parasite by an infected sand fly. Soon after their release, the MPs enter a phagocytic host cell. This study focuses on finding peptides that can inhibit MP-phagocytic host cell interaction.

Methods: We used a phage display library to screen for peptides that bind to the surface of *L. amazonensis* (causative agent for cutaneous leishmaniasis) and *L. infantum* (causative agent for cutaneous and visceral leishmaniasis) MPs. Candidate peptide binding to the MP surface and inhibition of parasite-host cell interaction were tested *in vitro*. Peptide Inhibition of visceral leishmaniasis development was assessed in BALB/c mice.

Results: The selected L. amazonensis binding peptide (La1) and the *L. infantum* binding peptide (Li1) inhibited 44% of parasite internalization into THP-1 macrophage-like cells *in vitro*. While inhibition of internalization by La1 was specific to *L. amazonensis*, Li1 was effective in inhibiting internalization of both parasite species. Importantly, Li1 inhibited *L. infantum* spleen and liver infection of BALB/c mice by 84%.

Conclusion: We identified one peptide that specifically inhibits *L. amazonensis* MP infection of host cells and another that inhibits both, *L. amazonensis* and *L. infantum*, MP infection. Our findings suggest a promising path for the development of new treatments and prevention of leishmaniasis.

KEYWORDS

phage display 12-mer peptide library, Leishmania-macrophage interaction, ligand-receptor interaction, metacyclic promastigote, visceral leishmaniasis

Introduction

Leishmaniasis is a neglected tropical infectious disease caused by *Leishmania*, a protozoan parasite transmitted by phlebotomine sand flies (World Health Organization [WHO], 2023). Leishmaniasis affects ~350 million people in 102 endemic countries, accounting for 1.3 million annual cases and 20,000 to 30,000 deaths every year

(Pan American Health Organization [PAHO], 2019). Over 20 *Leishmania* species can infect humans, manifesting through three main forms: cutaneous, mucocutaneous, and visceral leishmaniasis (Pan American Health Organization [PAHO], 2019; Mann et al., 2021; World Health Organization [WHO], 2023). Out of these, visceral leishmaniasis (VL) is the most devastating form, accounting for ~100,000 cases per year (Matlashewski et al., 2013; Pan American Health Organization [PAHO], 2019; CDC, 2020; Mann et al., 2021). Cutaneous leishmaniasis (CL) is the most common form and causes ulcer-like skin lesions on exposed parts of the body, leaving life-long scars, serious disability, and stigma. Currently, there are an estimated 700,000 to 1.2 million annual CL cases; the USA has been classified as endemic for leishmaniasis since 2015 due to autochthonous CL cases (McIlwee et al., 2018). Mucocutaneous leishmaniasis (ML) is caused by infection in the nasal and oral cavity, giving rise to strong inflammation and facial disfiguration (Pan American Health Organization [PAHO], 2019; Mann et al., 2021; World Health Organization [WHO], 2023). Only few treatments for leishmaniasis exist and can be toxic (Pan American Health Organization [PAHO], 2019; Mann et al., 2021; World Health Organization [WHO], 2023). Thus, the development of novel drugs and vaccines for leishmaniasis control is high priority. The treatment of leishmaniasis lacks a universal approach. Traditionally, antimonials administered through injections have been the primary therapy, but their use is associated with severe side effects. Liposomal amphotericin B is effective for certain forms of leishmaniasis but requires skilled administration. Miltefosine is the only available oral drug. Treatment guidelines vary across regions and depend on factors such as leishmaniasis type, causative parasite, patient immune status, and local therapeutic availability (eBioMedicine., 2023).

Vertebrate infection by *Leishmania* parasites is initiated by the delivery of flagellated motile MPs from the anterior gut of the infected sand fly to the bite site (Serafim et al., 2021). MPs enter phagocytic mononuclear cells at the bite site, such as neutrophils, monocytes, dendritic cells, or skin macrophages (Ueno and Wilson, 2012; Carneiro and Peters, 2021). Within the phagolysosome, MPs lose motility as their flagella retract during transforming into amastigotes, and then multiply and develop within the reticuloendothelial system. Amastigotes can travel through the circulatory or lymphatic system to cause mucosal or visceral disease (Ueno and Wilson, 2012; Carneiro and Peters, 2021). Therefore, MP infection of phagocytic host cells is the first obligatory step for disease development and a prime target for protection via immunization. Repeated probing by the female sand fly at the bite site causes laceration of capillary vessels and the formation of a blood pool which serves as the initial site of MP entry into host phagocytic cells. Tissue injury and sand fly derived factors (such as saliva, microbiome, and promastigote secretory gel) activate the release of chemokines that activate infiltration of host innate immune cells, such as neutrophils and monocytes (Serafim et al., 2021). Of note, MP infection of phagocytic host cells is the first obligatory step for disease development and a prime target for protection via immunization.

The final destination of parasites from each species is different, causing parasite species-specific disease forms. For instance, *L. infantum* develops VL, occasionally causing CL, but it is not associated with ML; *L. amazonensis* develops CL or ML, rarely VL (Carneiro and Peters, 2021; Mann et al., 2021). Although it is controversial whether MP entry into host cells is either passive or active (Rodríguez et al., 2006, 2011; Mayor and Pagano, 2007; Forestier et al., 2011; Walker et al., 2014; Andrade, 2019; Cavalcante-Costa et al., 2019; Valigurová and Kolářová, 2023), MP entry into phagocytic host cells is mediated by receptor-mediated phagocytosis (Carneiro and Peters, 2021). Other host cells and different *Leishmania* species display various surface molecules, leading to unique parasite-host receptor interactions for each [parasite species–host cell] pair (Ueno and Wilson, 2012). Moreover, MPs of a given species can interact with different receptors from different host cells, eliciting other downstream host cell functions (Ueno and Wilson, 2012; Carneiro and Peters, 2021). Therefore, characterization of the initial ligand-receptor interaction of each parasite species is likely to lead to mechanistic insights into downstream disease pathways (Carneiro and Peters, 2021; Mann et al., 2021). Molecular identification of ligand-receptor interaction for parasite-host cell recognition can be initiated by selecting peptides that interfere with this specific interaction (Ghosh et al., 2009, 2011; Cha et al., 2015, 2016, 2021; de Paula et al., 2022). Here, we used a phage peptide display approach to select peptides that bind to the surface of *L. amazonensis*, *L. infantum*, or both MP species, the parasite form responsible for initiating mammalian host infection. The selected peptides inhibited *in vitro* host cell internalization and *L. infantum* infection *in vivo*.

Materials and methods

Leishmania and THP-1 cell culture

Leishmania infantum (MHOM/MA/67/ITMAP-263) and *L. amazonensis* (MPRO/BR/1972/M1841-LV-79), parasites were cultivated at 26°C in Schneider's Drosophila Medium (Sigma S0146- with glutamine) supplemented with 10% FBS, 5% penicillin (100 U/mL)–streptomycin (100 μg/mL), and hemin (5 mg/mL) only for *L. infantum*.

THP-1 human monocyte cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and induced to differentiate into macrophages-like cells by treatment of PMA (Sigma-Aldrich; 1,000-fold dilution of a 0.1 mM stock in DMSO) and incubated for 4 days at 37°C (Auwerx, 1991; Traore et al., 2005; Cha et al., 2015).

Enrichment of MPs with Ficoll density gradient medium

Leishmania amazonensis and *L. infantum* were cultured for 8 days and then MPs were enriched by centrifugation using Ficoll-Paque® PLUS (GE Healthcare) gradient using 0, 10 and 20% solution. This step reduces contaminants, including debris and dead cells, without decreasing parasite viability and infectivity. MPs were quantified by hemocytometer counting following the protocol (Späth and Beverley, 2001). Parasite morphology was evaluated under light microscopy and were considered metacyclic promastigote when the flagellum length represents twice the size of body cell (da Luz et al., 2009; Sunter and Gull, 2017). Despite the purification, small amounts of other morphologies could be

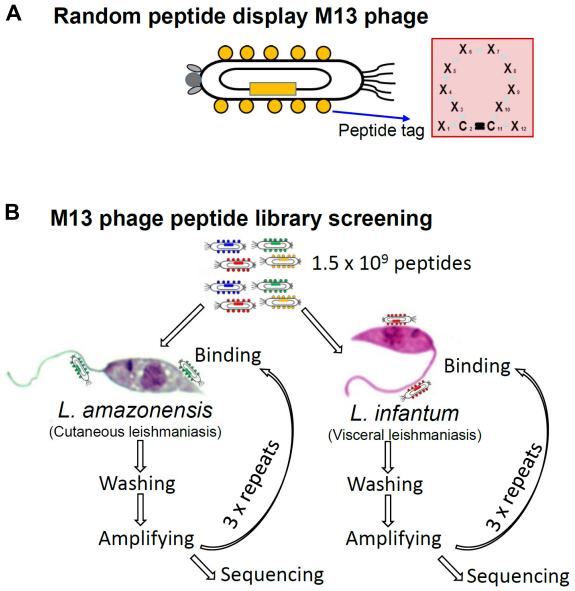

FIGURE 1
Structure of the phage peptide display library and strategy for screening parasite-binding peptides in search for *Leishmania* MP ligands that inhibit host cell interaction. **(A)** The M13 phage was engineered to display a random 12-amino acid peptides fused to the coat protein pVIII. The second and eleventh amino acids are fixed as cysteines to make a disulfide bond, which makes a circular conformation. **(B)** Library phages, of which complexity is calculated as 1.5×10^9, were incubated with enriched MPs of *L. amazonensis* or *L. infantum* for phages that inhibit MP-host cell interaction. After four repeated panning amino acid sequences of the selected peptide were deduced by DNA sequencing.

A

Species	ID	Sequence	Frequency (%)
L. amazonensis	La1	KCRQWWLDSRCG	4/31 (12.9)
	La2	RCRMWWTVHGCA	3/31 (9.7)
	La3	RCRTWWLPSPCH	2/31 (6.5)
	La4	RCRTWWLSPNCY	2/31 (6.5)
	La5	KCRHWWTTRGCT	2/31 (6.5)
	La6	RCRQWWIAGMCP	2/31 (6.5)
	La7	RCRKWWFQNMCA	2/31 (6.5)
L. infantum	Li1	ECKRARSAPNCN	2/29 (6.9)
	C3	-CASSSKSGPCP	1/29 (1.7)

-, stop codon

B

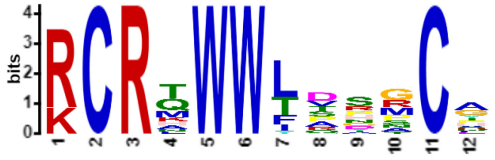

FIGURE 2
Predicted amino acid sequence of displayed peptides and analysis of the predominant amino acids at each position. **(A)** A total of 32 recombinant phages per species were sequenced after the fourth round of selection. All phages of the library have a cysteine at positions 2 and 11 (red font). One representing peptide of each species (bold red font) was selected for further experiments. Frequency was determined by the number of the selected phages that have an identical insert sequence out of the total number of successful sequences. **(B)** The La1 was selected using MEME analysis (https://meme-suite.org/meme/tools/meme).

included in the parasite pool. However, during our observations MP form was dominant.

Phage selection

In our study, we employed a filamentous M13 (f88.4) phage library that display 12 amino acid random peptide (Bonnycastle et al., 1996). Initial screening mixed 10^9 MPs with a total of 10^{11} library phages in 100 µl of culture medium. After 30 min incubation at 26°C, unbound and loosely bound phages were removed by washing with PBS six times. Bound phages were recovered by adding host *Escherichia coli* cells, followed by propagation of the phages in the added bacteria. This selection was repeated three more times, each time with the enriched phage population of the previous round. After the fourth round, the recovered phages were plated, and 32 random colonies were picked for sequencing of the DNA insert as previously described (Bonnycastle et al., 1996).

Synthetic La1, Li1, Li1scr peptide

La1 (biotin- KCRQWWLDSRCG), Li1 (biotin-ECKRARSAPNCN) and Li1scr (biotin-ACRKNRESNACP, the same amino acid composition as Li1 with a different sequence) peptides were synthesized by Peptide 2.0, Inc.

Phage/peptide binding assay and *in vitro* inhibition assay for MP-host cell interaction

We used separate titrations for phage/peptide binding assays (IFAs) and for inhibition assays. For phage/peptide binding assays, MPs of each species were fixed in 4% paraformaldehyde in PBS overnight at 4°C. Fixed MPs were blocked with 4% bovine serum albumin in PBS for 1 h to reduce non-specific binding. Subsequently, MPs were incubated either with 2×10^6 phages/µl or with 0.2 mg/ml of a synthetic peptide in PBS overnight at 4°C. After thorough washing steps, bound phages and biotinylated peptides were visualized using an anti-M13 phage antibody plus Alexa 488® -conjugated secondary antibody and Alexa 596® -conjugated streptavidin, respectively.

For *in vitro* inhibition assays, MPs of each species were fluorescently labeled using succinimidyl ester of carboxyfluorescein (CFSE) according to the manufacturer's instructions (Thermo Fisher Scientific). PMA treated THP-1 cells were prepared in an 8-well Lab-Tek II™ chamber slide (Nunc™) with 200 µl medium per chamber. CFSE labeled MPs were transferred into THP-1 cell culture with candidate phages (2×10^6 CFU/µl) or peptide (0.5 mg/ml). After 2 h incubation, the number of bound and internalized MPs were determined after triple washes the THP-1 cells with PBS. Using fluorescent microscopy, we observed that internalized MPs lost flagella to transform into a round amastigote form.

qRT-PCR assays

Total RNA was isolated from an infected mouse liver or a spleen using TRIzol® reagent (Thermo Fisher Scientific). The first-strand cDNA library was synthesized using Superscript III (Invitrogen) and random hexamers (Invitrogen). Relative parasite burden was determined by real-time PCR using a *Leishmania* specific primer set for LINJ_15_0010 histone H4 (Gene ID: 5067885): forward primer, 5′-GCTGAACCCGTCCGAGGT-3′; reverse primer, 5′-TGAGCCCTTTGCCGAACA-3′. *Leishmania* RNA quantity was normalized by mouse GAPDH expression as previously described (Cha et al., 2016). We used a total of 31 BALB/c mice. All animal experimental procedures were consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and were approved by the Mercer University Institutional Animal Care and Use Committee.

Statistics for data analysis

Two tailed Mann-Whitney *U*-test was used for non-parametric analysis in Figures 3–5.

Results

Screening a phage display library for peptides that bind to MPs

Seeking to identify peptides that bind to the surface of *Leishmania* MPs, we employed an M13 phage library (Bonnycastle et al., 1996) that displays random 12-amino acid peptides. The library has an estimated complexity of 1.5×10^9 different peptides. As shown in Figure 1A, library peptides have cysteines at positions two and eleven that form a disulfide bond that gives the peptides a circular conformation. We conducted separate screenings for peptides that bind *L. amazonensis* and *L. infantum* MPs. For initial screenings, 10^9 MPs of each species were incubated with a total of 10^{11} library phages and phages with low binding affinity were removed by thorough washing. Phages that remained bound to *Leishmania* MPs were recovered by adding host *E. coli* cells for phage amplification. After four rounds of panning, the peptide sequence displayed by the selected phages was determined via DNA sequencing (Figure 1B).

Properties of MP-binding peptides

Amino acid sequences displayed by phages enriched in the screen of both species are summarized in Figure 2A. A strong peptide enrichment in the *L. amazonensis* screen occurred, where 24 out of 31 sequenced phages displayed peptides with similar traits, having arginine and double tryptophan at the same positions (Figure 2B). One representative recombinant phage from each species was selected for further assays. For L. amazonensis the phage La1 represents 12.9% (4/31) of the total predicted sequences, while for L. infantum the sequence Li1 represents 6.9% (2/29). The C3 phage from the *L. infantum* screen was selected as a negative control, as it has a stop codon at the first amino acid position and is predicted to not display a peptide.

La1 and Li1 phages bind specifically to MPs and inhibit parasite internalization

To determine specificity of phage interaction with the surface of *L. amazonensis* and *L. infantum* MPs, we incubated La1 and Li1 phages with MPs of each species. Figure 3A shows that the La1 phage strongly binds to the *L. amazonensis* MP surface and not to *L. infantum* MPs. The Li1 phage binds to the *L. infantum* cell body only, not to the flagella. No binding above background was detected for the wild type (WT) and C3 phage controls. Specific binding of the selected phages to MPs raised two possible scenarios: (1) the phages bind to a putative MP ligand for host cell entry, or (2) the phages bind to an MP surface molecule unrelated to host cell interaction. In the first scenario, phage binding should result in inhibition of MP host cell entry, since phage occupancy of the ligand would preclude interaction with the presumed host cell receptor. In the second scenario, phage binding should not interfere with MP entry. To distinguish between the two possibilities, the selected phages were incubated for 2 h together with fluorescently labeled MPs and THP-1 human macrophage-like cell cultures. After washing with PBS, bound and internalized MPs were counted. Percent inhibition by the selected phages was determined by comparison with the control C3 phage-treated group. Figure 3B shows that the La1 phage selectively inhibit *L. amazonensis* MP-host cell interaction (47.4% inhibition). Interestingly, the Li1 phage inhibited both species, even though Li1 phage binding to *L. amazonensis* parasites was not detected in the experiments illustrated in Figure 3A. Li1 phages reduced the internalization of *L. amazonensis* MPs by 51.5% and *L. infantum* MPs by 53.8%. These results suggest that the selected phages bind to MP surface molecule(s) required for entry into macrophage cells.

Synthetic peptides La1 and Li1 inhibit parasite internalization

We also investigated whether inhibition of MP internalization was due to steric hindrance (phage particles have a diameter of 7 nm and a length of 1 μm) or by actual occupancy of MPs' ligand(s). These experiments were carried out with La1 and Li1 synthetic peptides and used a scrambled version of the Li1 synthetic peptide (Li1scr, ACRKNRESNACP) and the unrelated HP1 synthetic peptide (Cha et al., 2021) as negative controls (Figure 4). As shown in Figure 4A, both La1 and Li1 peptides bound to both parasite species. The binding of control peptides to MPs was limited to the parasite's body and was moderate compared to the La1 and Li1 peptide binding. To evaluate the potential inhibitory effects of the peptides, fluorescently labeled MP parasites were incubated with 0.5 mg/ml of each biotinylated synthetic peptide in a THP-1 human macrophage-like cell culture for 2 h. In line with the phage inhibition results, the La1 peptide selectively inhibited the internalization of *L. amazonensis* MPs into host cells by 44%, while the Li1 peptide inhibited the internalization of both

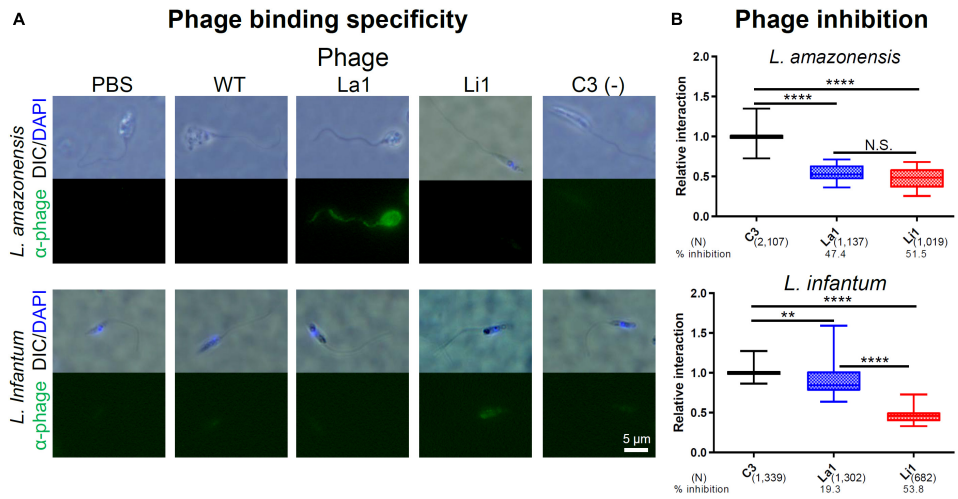

FIGURE 3
Selected phage binding inhibits parasite-host cell internalization *in vitro*. **(A)** Fixed MPs of each *Leishmania* species, denoted on the left were incubated with 2×10^6 CFU/µl of the selected phages denoted on the top. PBS, C3 phage, and wild type (WT) phage served as negative controls. After washing, bound phages were visualized with an anti-M13 phage antibody and Alexa 488® -conjugated secondary antibodies. **(B)** A total of 1×10^5 CFSE-labeled parasites were added to activated THP-1 macrophage-like cells in an 8-well slide monolayer culture (1 m.o.i.) together with 2×10^6 CFU/µL of selected phages denoted on the bottom. The number of attached and internalized parasites, in 100 microscopic fields under 600x magnification, were determined after 2 h at 37°C. Data pooled from five independent experiments. Percent inhibition was determined by comparing to C3 phage-treated group. *P*-values (**$P < 0.01$; ****$P < 0.0001$) were determined with the Mann-Whitney *U*-test. Vertical bars: range of the upper and the lower quartile, horizontal lines: medians, (N): number of MPs analyzed.

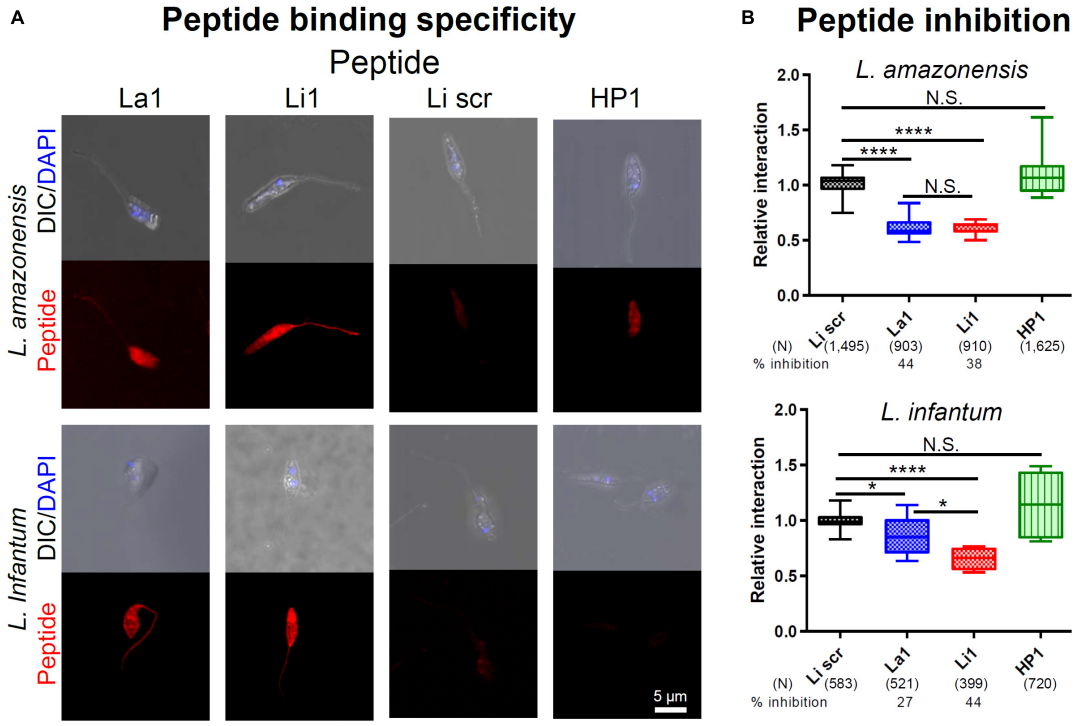

FIGURE 4
Selected synthetic peptide binding inhibits parasite-host cell interaction *in vitro*. **(A)** Fixed MPs of each *Leishmania* species, denoted on the left, were incubated in PBS with 0.2 mg/ml of the synthetic peptide denoted on the top. Li1scr and the unrelated HP1 peptide served as negative controls. After washing, peptide binding was visualized with an Alexa 596® -conjugated streptavidin. DAPI stained the parasite nuclei. **(B)** A total of 1×10^5 CFSE-labeled parasites were added to activated THP-1 macrophage-like cells in an 8-well slide monolayer culture (1 m.o.i.) together with 0.5 mg/ml of the peptide denoted on the bottom. The number of attached and internalized parasites, in 100 microscopic fields under 600x magnification, were determined after 2 h at 37°C. Data pooled from two independent experiments. Percent inhibition was determined by comparing to Li1scr peptide treated group. *P*-values (*$P < 0.05$; ****$P < 0.0001$) were determined with the Mann-Whitney *U*-test. Vertical bars: range of the upper and the lower quartile, horizontal lines: medians, (N): number of MPs analyzed.

species: 38% inhibition for *L. amazonensis* and 44% inhibition for *L. infantum*, respectively. The control HP1 peptide did not inhibit parasite-host cell interaction (**Figure 4B**). We conclude that steric hindrance does not play a role in inhibition by the recombinant phages and that the peptides are the effectors of inhibition of parasite entry into host macrophages.

Li1 peptide binding to *L. infantum* MPs inhibits VL development in BALB/c mice

To assess the potential *in vivo* inhibitory effects of the peptides on visceral infection and to corroborate *in vitro* findings, we performed *in vivo* inhibition assays. We incubated 1×10^7 *L. infantum* MPs with 20 μg of each peptide in 100 μl PBS (0.2 mg/ml) for 10 min at room temperature, and this mixture was injected into the peritoneum of BALB/c mice. Infected mice were sacrificed for RNA isolation from liver and spleen to determine parasite burden in each organ 30 days post infection. As shown in **Figure 5**, the Li1 peptide significantly reduced parasite growth in both organs: 84.7% inhibition in the spleen and 84% inhibition in the liver. La1 peptide has no inhibition of parasite growth in both organs as expected from the *in vitro* inhibition assays in **Figure 4B**.

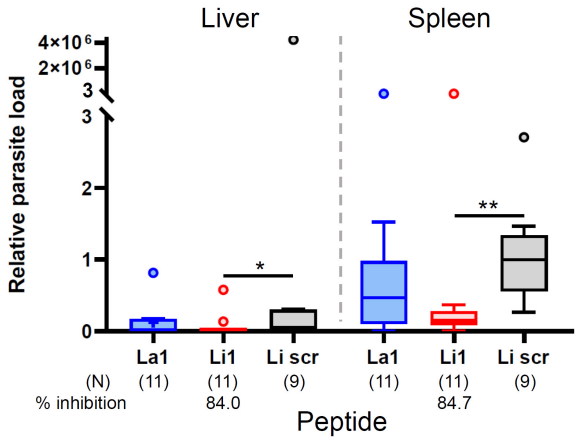

FIGURE 5
The Li1 peptide inhibits *L. infantum* infection and subsequent visceral leishmaniasis development. Ten million *L. infantum* MP parasites together with 20 μg peptide (denoted in the *X*-axis) in 0.1 ml of PBS were injected into the peritoneum of each BALB/c mouse. One month after injection, mice were sacrificed for quantification of the parasite load in liver and spleen. Parasite load was determined by qRT-PCR. Data pooled from three independent experiments. Each small circle denotes outliers by Tukey's analysis. Percent inhibition was determined by comparing to the Li1scr peptide treated group. *P*-values (*$P < 0.05$; **$P < 0.01$) were determined with the Mann-Whitney *U*-test. Vertical bars: range of the upper and the lower quartile, horizontal lines: medians, (N): number of mice analyzed.

Discussion

We have previously used a phage peptide-display approach using a filamentous M13 (f88.4) phage library to identify receptor-ligand interactions for malaria parasite invasion of the mosquito midgut, of the mosquito salivary glands and of the mammalian liver (Ghosh et al., 2009; Cha et al., 2015, 2016, 2021). Different from the phage libraries that display linear peptides, the f88.4 phage library displays circularized 12-amino acid peptides that maintain stable conformation. In this study, we screened the same phage library for peptides with high affinity to the surface of *L. amazonensis* and *L. infantum* MPs of which binding inhibits MP-host cell interaction. Although main surface protein GP63 has been reported to activate host cell phagocytosis (Brittingham et al., 1995), no parasite ligand and host cell receptor pair has been identified so far (Ueno and Wilson, 2012; Rhaiem and Houimel, 2016). Since *Leishmania* MPs can infect most phagocytic cell types we could not screen peptides for a specific host cell population, instead we screened peptides for homogeneous *Leishmania* MPs. As expected, we selected species-specific peptides from each screening. Given the complexity of the library (1.5×10^9), if the selection were not specific, the probability of isolating phages displaying the same peptide would be infinitely small. Thus, while the frequencies are not high at face value, the existence of phages displaying the same peptide isolated multiple times, indicate that such peptide must have affinity for the parasite. Along the same line of thought, the higher the number of phages displaying the same peptide that was selected, the higher the probability that such peptide has a higher affinity to the parasite. We observed that the diversity in phage enrichment may be attributed to differences in the parasite surface. We hypothesize that La1 and Li1 peptides are structural mimics of two different host cell surface molecules or two different epitopes on the same host cell surface molecule. Further studies using these peptides may provide insights into the differences between the two *Leishmania* species and their diverse clinical manifestations. Additionally, they can enhance our understanding of drug target specificity, distinguishing between species-specific and possible universal mechanisms, ultimately advancing our knowledge of *Leishmania* pathogenesis and treatment strategies.

Of note, La1 and Li1 peptides behave differently. Competitive inhibition assays using recombinant phages and synthetic peptides clearly confirm that La1 phage and La1 synthetic peptide selectively inhibit *L. amazonensis* MP-host cell interaction. On the other hand, Li1 phage and Li1 synthetic peptide inhibit MP-host cell interaction for both *L. amazonensis* and *L. infantum*. Although, phage binding assays do not show Li1 phage binding to *L. amazonensis* MP surface and Li1 phage binding to *L. amazonensis* MP surface, synthetic peptide binding assays clearly confirms the results of competitive inhibition assays using recombinant phages and synthetic peptides. Discrepancy of phage and peptide binding assays can be attributed to the size (~1 μm-long) of the phage particle. The extensive washing step can remove phage particles with moderate strength binding to the MP surface molecule. However, competitive inhibition assays co-incubate candidate phages and MPs in the host cell culture, and washing steps come after the incubation time. Therefore, washing steps do not remove phages on the MP surface, however, can remove weakly bound MPs on the host cell surface. Microscopic observation has shown that peptide binding does not inhibit MP mobility. Moreover, we observed that parasites infectivity was not affected during 8-h incubation of MPs with peptides for *in vitro* inhibition experiments (data not

shown), which implies that peptide binding does not affect parasite motility or viability, suggesting that inhibition is related to MP-host cell interaction.

Importantly our *in vivo* experiments confirm the results from *in vitro* inhibition assays: Li1 peptide binding to *L. infantum* MPs inhibits VL development in BALB/c mice, however, no inhibition was observed by La1 peptide. We inoculated MPs and peptide into the mouse peritoneum. Inhibition of 84% parasite growth in the mouse liver and spleen can be attributed to the initial inhibition of MP infection in the mouse peritoneum by Li1 peptide binding. We do not expect that the peptides affect the development of parasites inside the cells, as intracellular parasites have no access to the peptides. Therefore, the use of peptides for post infection treatment cannot be considered. Inhibition of VL development in the spleen was clearer than in the liver because parasite concentration was significantly higher in the spleen than in the liver (compare Li1scr treated groups in the liver and the spleen in **Figure 5**) since the proportion of phagocytic host cells in the spleen is higher than in the liver. Since Li1 peptide inhibits MP-host cell interaction for both species, it is expected that Li1 peptide mimics a common surface molecule on all phagocytic cells. On the other hand, La1 peptide may mimic a surface molecule of residential skin macrophages. As inhibition of parasite load using 0.2 mg/ml peptide was highly significant, we did not test the 0.5 mg/ml peptide treatment used for *in vitro* experiments.

In summary, we identified the La1 peptide that specifically inhibits L. amazonensis MP infection of host cells and the Li1 peptide that inhibits L. amazonensis and L. infantum MP infection. The Li1 specifically inhibits *L. amazonensis* MP infection into host cells and Li1 peptide that inhibits *L. amazonensis* and *L. infantum* MP infection. The Li1 peptide significantly attenuates development of leishmaniasis in mice. These findings open the way for the development of new strategies to prevent and treat leishmaniasis, an important neglected disease. Identification of the target molecules on the MP surface to which the La1 and Li1 peptides bind will lead to the identification of parasite ligands, which in turn, will serve as promising *Leishmania* vaccine candidates. The La1 and Li1 peptides may be applied for early intervention of MP infection at the sand fly bite site and further peptide application can be tested for amastigote parasite stage for inhibition of disease manifestation. Further, it will be important to assess the effectivity of these peptides to prevent infection by additional species of *Leishmania*.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by Institutional Animal Care and Use Committee, Mercer University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JV: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review and editing. MG: Validation, Writing – review and editing. MJ-L: Funding acquisition, Methodology, Resources, Supervision, Writing – review and editing. S-JC: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and 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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Uncovering the diversity of pathogenic invaders: insights into protozoa, fungi, and worm infections

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Post COVID-19, there has been renewed interest in understanding the pathogens challenging the human health and evaluate our preparedness towards dealing with health challenges in future. In this endeavour, it is not only the bacteria and the viruses, but a greater community of pathogens. Such pathogenic microorganisms, include protozoa, fungi and worms, which establish a distinct variety of disease-causing agents with the capability to impact the host's well-being as well as the equity of ecosystem. This review summarises the peculiar characteristics and pathogenic mechanisms utilized by these disease-causing organisms. It features their role in causing infection in the concerned host and emphasizes the need for further research. Understanding the layers of pathogenesis encompassing the concerned infectious microbes will help expand targeted inferences with relation to the cause of the infection. This would strengthen and augment benefit to the host's health along with the maintenance of ecosystem network, exhibiting host-pathogen interaction cycle. This would be key to discover the layers underlying differential disease severities in response to similar/same pathogen infection.

KEYWORDS

pathogens, protozoa, fungi, worms, host-pathogen interaction, immune evasion

1 Introduction

The disciplines of agriculture, environmental science, and medicine all greatly benefit from the study of fungi, worms, and detrimental as well as beneficial protozoa. These groups contain pathogenic species that can infect people, animals, and plants; thus, it is essential to study them for strengthening agricultural production and public health. On the other hand, beneficial fungi, worms, and protozoa have a vast range of uses, including the biocontrol of pests, the bioremediation of contaminated environments, and the production of industrial enzymes and pharmaceuticals. The motivations behind the conception of studying both pathogenic and useful organisms lie in the dual pursuit of mitigating health threats and harnessing the beneficial aspects of sustainable development. By understanding the complexities of these microorganisms, researchers will gain information to improve agricultural methods as well as develop focused approaches for disease prevention and treatment, and they will be able to unleash the full potential of these organisms to provide beneficial gains to human health and the environment. This thorough understanding is necessary for advancing fields that directly affect food security, environmental protection, and human health.

2 Protozoa

A class of organisms known as protozoa inhabit this hidden domain of extraordinary diversity and complexity where the distinction between microorganisms, plants, and animals is hazy. The Greek words "Protos," which means first, and "zoa," which means animals, are the source of the word "protozoa. "Protozoa" are largely free-living creatures that first appeared on Earth about 1.5×10^9 years ago. Protozoa have a highly organized anatomy and carry out sophisticated metabolic processes. It has been noted that these organisms possess a variety of specialized organelles that enable them to move, eat, and reproduce. Some are seen to have whip-like flagella, and others exhibit a kind of locomotion by extracting and retracting pseudopodia (Yaeger, 1996; Zambrano-Villa et al., 2002). Organisms belonging to superior levels of classification hierarchy are observed to be infected with either a single or multiple species of protozoa. Disease conditions may vary subject to the type of the infectious species and can be asymptomatic or in some cases can also be life-threatening. Despite their diminutive size, these microorganisms provide a profound influence on the natural world both as agents of disease and as symbiotic partners (Zheng et al., 2020). Hence, from understanding the mechanism of disease transfer to harnessing the potential of beneficial protozoa the significance of these microscopic organisms extends far from their size.

2.1 Protozoa as disease agents

Among a plethora of protozoa, a subset of these microscopic organisms has acquired the ability to infect and further cause disease in humans and animals. These pathogenic strains have evolved complex mechanisms for invasion and survival in the host. Protozoa frequently go through several sequential stages, that provide them the flexibility to take advantage of various tissues or hosts as they make their way through the challenging transition from existence to transmission, and the sickness often results in tissue damage and subsequent illness (Zheng et al., 2020; Akoolo et al., 2022). An assortment of symptoms, from fever and anaemia to cognitive dysfunction and organ failure, can result from the chronic infectious disorders caused by infectious protozoan species that impact the blood, gastrointestinal system, liver, and brain (Seed, 1996). Some of the examples of disease-causing protozoa include *Entamoaeba histolytica, Giardia lamblia* (intestinal parasitic protozoa), *Trichomonas vaginalis* (a urogenital disease in female individuals), *Naegleria fowleri* (meningoencephalitis), *Trypanosoma cruzi* (American Trypanosomiasis or Chagas disease) and *Plasmodium* spp. (Malaria) (Fürnkranz and Walochnik, 2021; Reyes-López et al., 2022) ()thus representing an important set of pathogenic protozoans posing some serious infectious threats among a variety of hosts, including humans.

2.2 Mechanism of protozoan pathogenesis by immune evasion strategies and alternative strategies

Protozoa reduce humoral responses, in which antibody–antigen complexes form at the site of excess antibody presence and activate factor XII, also known as Hageman Blood Coagulation Factor, which in turn activates the kinin, fibrinolytic, and complement systems. Additionally, this type of hypersensitivity is linked to several complications, such as edoema, blood hyperviscosity, and hypotension. Similar kinds of humoral immune responses are also observed in other infections caused by protozoa that are listed in Table 1 (Gurung and Kanneganti, 2016; Sardinha-Silva et al., 2022).

In addition to this, some of the most pivotal immune evasion mechanisms utilized by various pathogenic protozoa to infect the host system are majorly observed to be categorized into 5 types:

2.2.1 Intracellular localization

Numerous protozoan parasites proliferate and expand inside the host cells. *Leishmania* and *Toxoplasma*, for example, are known to develop in macrophages, but *Plasmodium* is known to grow in hepatocytes first, then in red blood cells. A portion of the pathogen's life cycle may be spent inside cells, where it is shielded from both intracellular digestion and the lethal response of lymphocytes and antibodies. For instance, it has been found that the extracellular sporozoite and merozoite stages of the life cycle of the protozoan parasite *Plasmodium* are susceptible to the antibody response. Other than this, it has been noted that *Trypanosoma cruzi* can break free from the phagocytic vacuole and enter the cytoplasm of the macrophage (Sibley, 2011; Graewe et al., 2012).

2.2.2 Antigenic masking

The pathogen encloses itself in the host's contents during antigenic masking, allowing it to escape and be identified as foreign. For instance, the surface of several species of trypanosomes is attached to host immunoglobulins. These immunoglobulins block the parasite from being recognised by the host molecules' immune systems because they are not linked to the variable region of the molecule, most likely through the Fc fragment (Chulanetra and Chaicumpa, 2021).

2.2.3 Blocking

When antigen–antibody complexes in the serum of infected animals attach to the surface of the pathogen, they impede the pathogen's ability to elicit a response from lymphocytes and cytotoxic antibodies, so precisely limiting the activity of lymphocytes directed towards the pathogenic organism (Chaplin, 2010). This kind of immunological escape mechanism has been proposed for parasite helminths and cancer cells. Figure 1 depicts the various immune evasion strategies employed by the pathogenic protozoa inside the human host.

2.2.4 Immunosuppression

In immunosuppression, the host's immune activity is lowered either towards a pathogen specifically or in a generalised manner towards a

TABLE 1 Describing some pathological mechanisms associated with pathogenic protozoa.

Parasitic protozoa	Pathological Mechanism
Malaria	Mechanical tissue damage
African Trypanosomiasis, Malaria	Immunosuppression
African Trypanosomiasis, Malaria	Immediate type hypersensitivity
American Trypanosomiasis	Autoimmunity
Leishmaniasis, Toxoplasmosis, Amebiasis	Delayed type hypersensitivity

foreign antigen. With context to protozoan infections, a number of mechanisms have been proposed that include: (i) The presence of the host components that non-specifically activate the proliferation of specific anti-parasitic B cells. (ii) Production of a particular immune suppressor component by the pathogen. (iii) The generation of regulatory cytokines by suppressor T-cells or macrophages, which impair the immune system (Dupont et al., 2012; Saleki et al., 2023).

Due to immunosuppression, a few microorganisms may be able to evade the immune system and develop a persistent infection. Because it allows for the undiscovered presence of a few parasites bearing novel surface antigens, this kind of mechanism may be useful for infections undergoing antigenic change (Finlay and McFadden, 2006). Experimentally induced immunosuppression by a number of external components has been observed to produce elevated parasitism, higher infection rates or both. Other than this, immunosuppression is taken to be pathogenic itself as it has been observed that a lowered response towards heterologous antigens could be advantageous for secondary infections and in turn these infections may often be involved in death as seen in the case of *African trypanosomiasis* (Evering and Weiss, 2006).

2.2.5 Antigenic variation

During an infection, some pathogenic protozoans change their surface antigens, a process known as antigenic variation, which helps them avoid being recognised by the host's immune system. It has been determined that *Babesia*, *Plasmodium*, and *Giardia* are three significant groups of pathogenic protozoa that can change the antigenic characteristics of their surface coat (Dzikowski and Deitsch, 2006; Chulanetra and Chaicumpa, 2021). For example, *African Trypanosomes* can fully substitute the antigens on their surface glycocalyx every time the host exhibits a new humoral response. It has been documented that approximately one thousand genes encoding surface antigens can be found in African trypanosomes. For these genes to be active, they must be positioned in the telomeric region of the chromosome, even though they are located on various chromosomes. Furthermore, a multitude of separate gene families that encode outer surface proteins in *Giardia* have been identified and suggested to aid *Giardia* in evading the immune response of the host through the mechanism of antigenic diversity (Nash, 1997; Faubert, 2000; Lee et al., 2021). The apicomplexan parasites of the genus *Plasmodium* develop in erythrocytes in repeated cycles during malaria infection. In addition to variations in antigenic diversity within a strain, the species *Plasmodium falciparum* exhibits strains that differ in many polymorphic proteins. The *Plasmodium falciparum*-infected erythrocyte membrane protein 1 (PfEMP1) antigens, which are expressed on the surface of the infected erythrocytes, are the finest known example of genuine antigenic variation. Through altering the expression of PfEMP1, the parasite circumvents the immune reaction aimed at these immuno-dominant antigens. The PfEMP1 proteins also prevent dendritic cells from presenting antigens and enable infected red blood cells to adhere to the endothelium and extracellular matrix, preventing the spleen from eliminating the infected erythrocytes. PfEMP1 variable proteins have a molecular weight between 200 and 350 kDa. A range of endothelial receptors, including CD36, vascular cell adhesion molecule-1 (VCAM-1), E-selectin (ELAM-1), and intercellular cell adhesion molecule type 1 (ICAM-1), as well as the extracellular matrix protein thrombospondin, can be found in their extracellular region. These variable adhesive domains give the parasite-infected erythrocytes a specific binding specificity. The clinical signs of malaria are determined by the adhesive characteristics that cause infected erythrocytes to be sequestered in the liver, kidneys, brain, lungs, or other organs.

Similar to *Plasmodium*, *Babesia* is an intraerythrocytic parasite that is spread by ticks rather than mosquitoes. Clonal antigenic

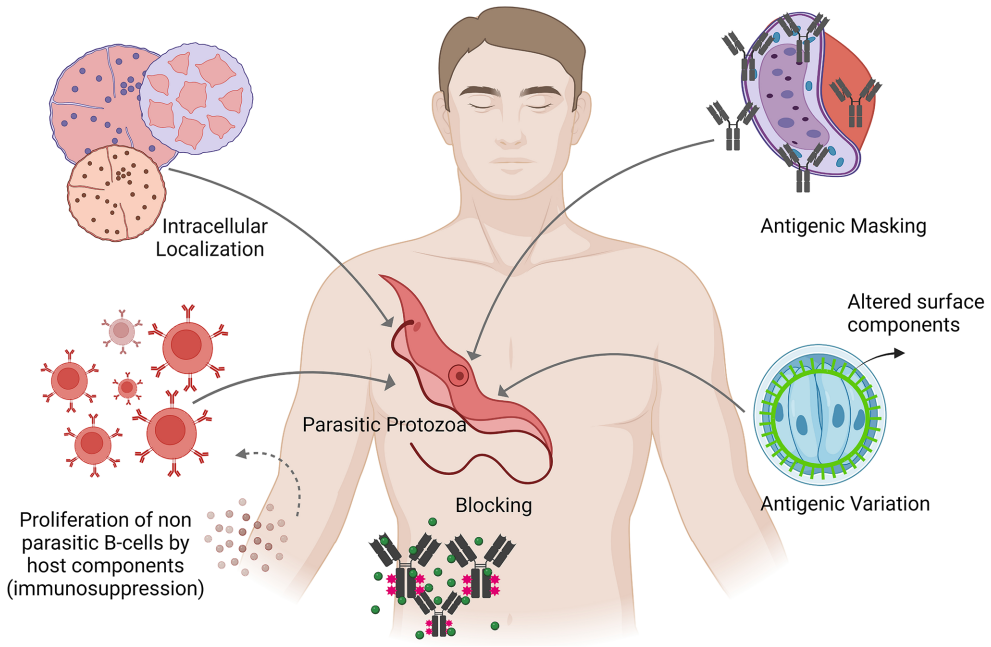

FIGURE 1
Depicts the various immune evasion strategies employed by the pathogenic protozoa inside the human host.

variation of the bovine parasite *Babesia bovis* is the most reported variation, even though multiple multigene families have been described for different species of *Babesia*. The surface of infected red blood cells expresses a heterodimeric protein known as the variable erythrocyte surface antigen (VESA1) of *Babesia bovis*. These polymorphic proteins very quickly, which probably prolongs the parasite's life through immune evasion and sequesters the infected red blood cells in peripheral organs, leading to chronic infection in cattle. With an estimated molecular weight of 128 kDa, the VESA1 proteins are expressed on the outermost tips of the membrane knobs in infected erythrocytes. The VESA1 proteins' structural alterations and antigenic modifications determine their cytoadhesive behaviour (Barbour and Restrepo, 2000).

2.2.6 Prevention of phagocytosis

By altering its interaction with host phagocytic receptors as well as controlling downstream signaling cascades, *Plasmodium* species inhibits phagocytosis. For instance, *Plasmodium yoelii* primarily infects erythrocytes that express high levels of CD47 (marker responsible for preventing phagocytosis), thereby helping them avoid being phagocytosed by the splenic red pulp macrophages. Additionally, through modifications to complement regulatory proteins that insulate infected host cells from complement-mediated damage, parasites prevent phagocytosis. They deactivate C3b on the surface of infected erythrocytes, impeding complement-mediated phagocyte removal of the parasites (Cristina Vanrell and Silvia Romano, 2023).

2.2.7 Resistance to oxidative response

In the case of *Trypanosoma cruzi*, reduction of ONOO synthesis in NO-exposed parasites, regulation of NO-exposed parasites, and protection from the direct lethal effects of O2/H2O2 on parasite mitochondria within the macrophage phagosome are few of the strategies that the parasite employs to resist the deleterious effects of oxidative responses from the host. *T. cruzi* possesses a plethora of detoxifying antioxidant defenses and redox metabolism for protection against host-derived oxidants. One of the most significant thiols utilized by the trypanosomatid-antioxidant system is trypanothiol (T[SH]2). Additionally, *T. cruzi's* Fe-dependent superoxide dismutases (Fe-SODs) efficiently eliminate O2 and may aid in intracellular survival (Estrada et al., 2018). *T. cruzi* has also been reported to harbour TcAPxCcP, a type A hybrid peroxidase that uses cytochrome C and ascorbate as reducing substrates for H2O2 elimination. TcAPxCcP is a membrane-bound peroxidase that is present in the mitochondria and endoplasmic reticulum as well as plasma membrane during the parasite's life cycle (Hugo et al., 2017). On the whole, *T. cruzi's* antioxidant defense mechanism detoxifies reactive species in the phagosomal compartments, which contributes to its virulence.

2.2.8 Formation of a distinct vacuole to hinder host defense

In this case, the organelle harboring *Toxoplasma gondii* appears to be arrested and unable to merge with lysosomes, hence protecting host-defense system and successful survival of the parasite. Apart from this, *T. gondii* demonstrates ways by which intracellular pathogens employ certain components of the host pathway (for instance Rab-family GTPases) for nutrient transportation to promote the proliferation of the pathogen (Robibaro et al., 2001; Paone and Olivieri, 2022).

2.2.9 Uptake of hemoglobin from the host

Hemoglobin and ferritin (specific intracellular proteins) are the main cellular reserves of iron in mammals. Erythrocytes contain hemoglobin (Hb) which is a vital supply of iron and amino acids for pathogenic protozoa to flourish inside the infected host. For instance, in the case of *Giardia lamblia* (an intestinal protozoan parasite), the parasite is observed to exhibit lysosomes as peripheral vacuoles with hydrolase and acid phosphatase activity that might be pivotal in cell feeding and excystation. The ideal pH range for these proteinases to function against Hb is 3.5 to 7.0; hence, protease secretion directed against Hb influences host interactions. In addition to this, in the event of *Trichomonas vaginalis*, a certain percentage of the reason trichomonads are successful parasites of the vaginal epithelium is linked to their ability to take in vital nutrients like iron. Since, in addition to controlling cytoadherence, cytotoxicity, hemolysis, complement resistance, immunological evasion, and apoptosis in human cells, iron likewise impacts the virulence of *T. vaginalis*. As there is no free iron in the vaginal environment, *T. vaginalis* obtains iron from host proteins that either bind to iron or contain iron, including cytochromes, Lactoferrin, and Hb. When the amount of lactoferrin decreases during menstruation, hemoglobin (Hb) in erythrocytes serves as a significant source of iron. Hemolysis and erythrophagocytosis are the two main methods that *T. vaginalis* uses to obtain Hb iron *in vivo*, which serves to aid in the growth and differentiation of the parasite inside the host (Reyes-López et al., 2023).

2.3 Protozoan diseases in humans

2.3.1 African trypanosomiasis (sleeping sickness)

Human African trypanosomiasis (sleeping Sickness) is a vector-borne parasitic disease. The disease is transmitted to humans by bites of *Glossina* (tsetse fly) which have acquired the disease-causing protozoa from infected animals or humans. Pathogenic protozoa take two forms depending on the parasite subspecies called as *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. With a tiny kinetoplast and a well-developed undulating membrane, these two *T. brucei* subspecies are morphologically very similar. While in the blood or cerebral fluid, parasitic protozoa multiply. A feeding fly ingests trypanosomes, which then enter the salivary glands and reproduce rapidly as epimastigotes attach to the gland's microvilli until they modify into metacyclic trypomastigotes found in the lumen. About 15 to 35 days after infection, the fly becomes contagious for humans. The disease is mainly transmitted through tsetse fly however there are other pivotal possible ways of disease transmission like: (i) Mother to child: Trypanosomes can cross the placental barrier and hence can infect the foetus. (ii) Infection in laboratories via pricks with contaminated needles. (iii) Transmission through sexual contact.

2.3.1.1 Clinical features

With context to the clinical attributes associated with the disease the parasite is initially observed to be multiplying in lymph, blood, and subcutaneous tissue, called the hemo-lymphatic stage (first stage), leading to symptoms like headache, fever, itching, joint pain, and enlarged lymph nodes. Following this, the pathogen crosses the blood–brain barrier and enters the nervous system, causing the meningo-encephalitic stage (second stage), which displays signs including sensory commotion, behavioural changes, sleep-cycle

disturbance, and poor coordination. In addition to this, when a patient has *Trypanosoma brucei rhodesiense* infection, they often have an early symptom of regional lymphadenitis; however, this is less common in people with *T. b. gambiense* infection. Following a phase of localized growth, the trypanosomes penetrate the bloodstream through the lymphatic system, potentially causing recurrent fever, headaches, lymphadenopathy, and splenomegaly. Subsequently, somnolence, cachexia, coma, and death occur, along with indications of meningoencephalitis. Enlargement of the posterior cervical chain of lymph nodes, also known as Winterbottom's sign, is more frequently caused by a *T. b. gambiense* infection (Kennedy and Rodgers, 2019).

2.3.1.2 Pathogenesis and host defense

In order to survive in hosts that are continuously infected, African trypanosomes have developed incredibly complex evasion strategies, including antigenic mutations, excessive complement system activation resulting in chronic hypocomplementemia, downregulation of nitric oxide generation, polyclonal B-lymphocyte activation, and severe immunosuppression (Bezie, 2014). Inflammatory changes leading to demyelinating encephalitis are observed as the disease progresses. Associative infections like pneumonia have been proposed to be caused by the parasite's membrane's immunosuppressive response. In addition to this, the liberation of common antigens in each episode of trypanolytic crisis (trypanosome lysis) leads to antibodies as well as cell-mediated hypersensitivity reactions. At the site of inoculation, inflammatory reactions occur, along with regional lymphadenitis. Inflammation in the brain and heart also develops, accompanied by a cell-mediated immune reaction along with higher levels of IgM.

2.3.1.3 Diagnosis and control

The pathogen can be found in the blood and lymph nodes of the human host infected early in the disease's course, and subsequently in the host's cerebrospinal fluid. The Gambian form of the infection appears to be persistent and takes years to develop CNS-related abnormalities, whereas the Rhodesian variant develops more acutely and eventually results in death. For the proper disease diagnosis laboratory animal inoculations or cultures are found to be useful. Apart from this, serological tests including indirect hemagglutination and indirect immunofluorescence are also found to be advantageous for disease detection. In terms of control methods, pesticides and traps have been used to regulate tsetse fly populations. Apart from this, medications like melarsoprol and eflornithine (difluoromethyl-ornithine) are used to treat severe stages of illness conditions, while pentamidine and arsenical suramin are used to treat early phases of infection.

2.3.2 *Trichomonas vaginalis*

Trichomonas vaginalis is a pathogenic protozoan responsible for causing trichomoniasis, i.e., the most widespread, non-viral sexually transmitted infection (STI). *T. vaginalis* is a motile protozoan species that inhabits the lower genitourinary tract of females and prostate and urethral regions in males (Petrin et al., 1998; Menezes et al., 2016). The concerned disease is observed to elevate the risk of Human Immunodeficiency virus (HIV) transmission in both males and females. Other than this, the disease is also linked with detrimental outcomes during pregnancy. The pathogen possesses a pear-shaped body organization, a single anterior nucleus and a single posterior flagellum that constitutes the exterior border of an undulating membrane. The life cycle of *Trichomonas* is quite uncomplicated as it remains only as a single trophozoite form and since there is the absence of any resistant cyst-like stage the mode of transfer from host to host is simply direct. The various types of risk factors associated with the infection (Beri et al., 2019). It includes association with an infected partner, multiple sex partners, person associated with any history of Sexually Transmitted Infections (STIs), drug abuse, and avoiding usage of any barrier contraception.

2.3.2.1 Clinical Features

Trichomonas infection in females is generally chronic and is represented primarily by dysuria, vaginitis and vaginal discharge The swelling of the vagina is generally spread out and is represented by hyperemia of the vaginal wall along with the movement of polymorphonuclear leukocytes into the vaginal lumen Most women (85%) and men (77%), who are infected with *Trichomonas vaginalis*, do not exhibit any symptoms. In less than 6 months, one-third of asymptomatic women develop symptoms. The urethra, the vagina, and the endocervix are common places for infections in women. The pH of the vagina is normally 4.5, but when there is a *Trichomonas vaginalis* infection, it frequently rises to >5. Approximately, 5% of women experience *coplitis macularis*, also known as strawberry cervix; however, this number increases to almost 50% following colposcopy. Adnexal, endometrial, Skene and Bartholin gland infections are among the other side effects. *Trichomonas* infection can result in decreased sperm cell motility, prostatitis, and epididymitis in males (Kissinger, 2015). Figure 2 interprets the various associated outcomes with response to *Trichomonas vaginalis* infection.

2.3.2.2 Pathogenesis and host defense

Regarding the pathogenesis of *Trichomonas vaginalis*, all pathogenic mechanisms (i.e., immune response, contact-dependent, and contact-independent) are likely relevant in the virulence of this illness. Four adhesion proteins – AP65, AP51, AP33, and AP23 – seem to be involved in the parasite's attachment to epithelial cells. Their specific receptor-ligand interactions are influenced by pH, temperature, and time. The host cell receptors that the parasite adhesion molecules attach to are not well understood; however, there is some indication that laminin may be a potential target for trichomonad adherence. Conversely, it seems that the parasite's virulence and hemolytic activity are connected. Considering that *T. vaginalis* is incapable of synthesizing lipids, erythrocytes could be a major supply of the fatty acids the parasite requires. Furthermore, the lysis of red blood cells might provide the parasite with iron, a crucial nutrient. Moreover, a wide range of hydrolases, with cysteine proteinases being particularly common, have been identified in *T. vaginalis*. Apart from this, *Trichomonas* symptoms are frequently worse during menstruation; this fact may be explained by the functions that pH and hormones play in the disease. Menstrual blood raises the pH above what is typically found in the vagina, providing a rich environment for *T. vaginalis* proliferation. Furthermore, more iron is made available by the blood, which improves *T. vaginalis*' capacity to adhere to the vaginal epithelium (Preethi et al., 2011). Additionally, the reason for the inflammation reaction in the case of trichomoniasis is not completely clear, however, one of the reasons suggested for the condition is mechanical irritation occurring from contact between the vaginal epithelium and the parasite. Research

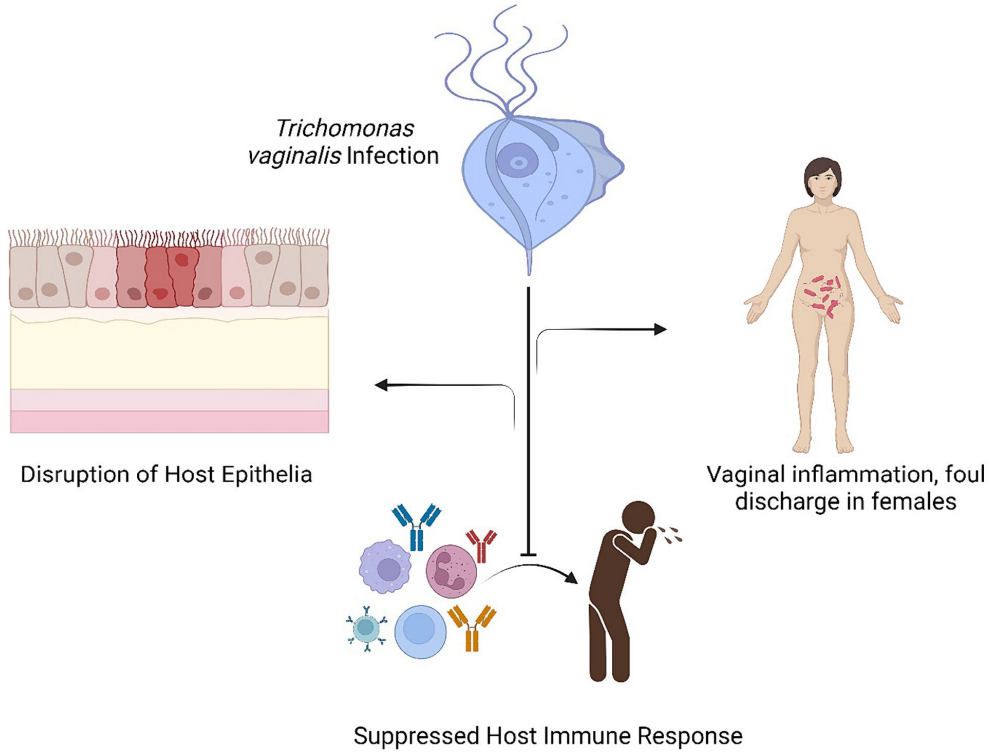

FIGURE 2
Interprets the various associated outcomes with response to *Trichomonas vaginalis* infection.

investigations using animals suggest the protective role of antibodies in overcoming signs and symptoms associated with the infection. However, the antibody response activated during the infection is short-lived and disappears completely in 6 to 16 months (Zhang et al., 2023).

2.3.2.3 Diagnosis and control

Typically, a wet mount preparation of discharge from the patient is tested for diagnosing *T. vaginalis* infection. The presence of pear-shaped trophozoites, typically 7 to 23 μm in length with "bobbling."

2.3.3 Malaria

Malaria is a parasitic disease transmitted by *Anopheles* mosquito that causes acute life-threatening condition and poses a serious global health threat. The multistage developmental process of *Plasmodium* causes the infected host to have periodic fever bouts. With consideration to the life cycle of the pathogenic protozoan, *Plasmodium* undergoes two stages of development: an asexual stage in the human host and a sexual phase in the *Anopheles* mosquito, which serves as the carrier. Within the liver parenchymal cells, where they proliferate asexually, the sporozoites that are delivered during a blood meal quickly enter the bloodstream (a phase called as schizogony). The asexual reproduction of protozoa is known as schizogony, previously known as merogony. A schizont is made up of many cell nuclei; the daughter nuclei organize into single entities called merozoites when they are encircled by cytoplasm. Between 10,000 and over 30,000 merozoites can be produced by a single sporozoite. The enlarged liver cell bursts and discharges the mobile merozoites into the bloodstream when schizogony gets completed. These merozoites stick to the red blood cells through certain surface receptors (glycophorin A in the case of *P. falciparum*, or Duffy blood group antigen, Fya or Fyb, in the case of *P. vivax*). Following their entry into red blood cells, they develop into trophozoites. By the time the 48-to 72-h erythrocytic phase concludes, the red blood cells' schizonts get developed. Vacuoles with parietal nuclei, often known as seal-ring forms, might develop during this period. Red blood cells that have degraded may release fresh merozoites that can infect more red blood cells. Within erythrocytes, a portion of the merozoites evolves into sexual phases, creating macro-and microgametocytes. The malaria pigment, an insoluble metabolite of hemoglobin, forms hemozoin in the intra-erythrocytic vacuoles. The *Anopheles* mosquito's midgut produces a motile, flagellated zygote following the uptake of both male and female gametocytes during a blood meal. This zygote enters the gland that produces saliva. Finally, an oocyst forms, generating sporozoites that can spread to a new human host through the mosquito's saliva (Arbeitskreis Blut, Untergruppe «Bewertung Blutassoziierter Krankheitserreger», 2009; Cox, 2010; Venugopal et al., 2020).

2.3.3.1 Clinical features

The most common symptom associated with the infection is fever. Other major signs related to malaria include vomiting, nausea, myalgias, diarrhea, chills, abdominal pain and headache. With the advancement of the disease, some cases are observed to develop the classic malaria paroxysm that consists of 3 successive stages:

a Stage 1 (Cold stage): 15–60 min characterized by a feeling of cold and shivering.

b Stage 2 (Hot stage): 2–6 h consisting of fever (around 41°C), nausea, vomiting, flushed dry skin and headache.
c Stage 3 (Sweating stage): 2–4 h. Fever drops promptly and the patient sweats (Nureye and Assefa, 2020).

In addition to this, various other important complexities associated with the infection include cerebral malaria, nephrotic syndrome (NS) and severe malarial anaemia. Other than this some pivotal added complications associated with malaria are:

a Algid Malaria, an adrenal deficiency that occurs due to pathogenic blockage and associated necrosis of the adrenal gland.
b Bilious remittent fever characterized by abdominal pain and constant vomiting that may cause jaundice, dark urine and dehydration.
c Circulatory collapse, acute respiratory distress syndrome, pulmonary edema, intravascular coagulation, coma and death (Vandermosten et al., 2018).

In an instance of simple malaria, several laboratory abnormalities could be observed. The conditions that fall under this category are proteinuria, increased liver and renal function tests, thrombocytopenia, leukocytosis or leukopenia, hypoglycemia, hyponatremia, and laboratory evidence of disseminated intravascular coagulation. Rarely, patients with severe malaria may exhibit hemoglobinemia and hemoglobinuria together with significant intravascular hemolysis. Additional risks include shock, pulmonary, cardiac, hepatic, or renal failure, convulsions, hyperparasitemia (greater than 3–5% of the RBCs get parasitized), prolonged hyperthermia, severe hypoglycemia, lactic acidosis, spontaneous bleeding, high output diarrhea or vomiting. Splenic rupture, aspiration pneumonia, and gram-negative sepsis are further malarial infection consequences (Idro et al., 2005).

2.3.3.2 Pathogenesis and host defense

Clinical symptoms are associated with the erythrocytic stage of the pathogen. Primary symptoms of malaria are associated with erythrocyte rupture, which sets off the host's immunological response. Reactive oxygen intermediates, cytokines, and other substances generated during immunological activity are thought to be important players in pathogenesis, causing fever, chills, weakness, and other infection-related systemic symptoms. With context to *Plasmodium falciparum*, infected red blood cells stick to the endothelium of capillaries and post-capillary venules causing blockage of microcirculation and local tissue anoxia. In other organs including the kidney, intestine and brain the pathogen is observed to be responsible for causing acute tubular necrosis, ulceration and cerebral malaria, respectively (Clark et al., 2004; Joos et al., 2010). Apart from this, the pathophysiological basis of severe malaria manifestations, including brain malaria, is believed to be parasite sequestration. It impairs blood flow, which results in localized hypoxia. It promotes the growth of parasites and the ability of infected RBCs to adhere to healthy red blood cells. Furthermore, when parasites sequester, the effects of their toxins are more restricted, and the host immune response is also stimulated, perhaps leading to targeted synthesis of inflammatory mediators and tissue damage. RBC and infected RBC thus become less malleable and stiffer. Sequestration occurs when parasites attach themselves to the placenta during gestational malaria. The primary adhesion receptor, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), binds to the trophoblastic villous endothelium mostly through chondroitin-4-sulfate (CSA) and other sugars such as glycosaminoglycans as well as hyaluronic acid (HA). In particular, during the first pregnancy, when women typically lack adequate antibodies against CSA-binding parasites, malaria during pregnancy can be extremely dangerous for mothers and result in fetal death (Autino et al., 2012). In view of the host response, malaria is proposed to be regulated by acquired as well as hereditary factors. For example, the lack of *P. vivax* infection in African regions can be explained through the absence of Duffy-blood group antigens, which *P. vivax* merozoites bind to. Ovalocytosis, which is common in some malaria-prone areas like New Guinea, has been suggested to potentially lower the incidence of malaria since malarial infections do not grow in ovalocytes. Aside from this, cytotoxic T-cell response, which is used to counteract the pathogen's liver stages, and naturally developed immunity, which involves cytokine release, both function to guard against all stages of the infection (Rodrigues et al., 2014).

2.3.3.3 Diagnosis and control

Direct pathogen observation in thick and thin blood smears stained with Giemsa is typically required for a specific diagnosis of malaria. Thin blood smears in which the parasite is visible inside the red blood cells are used to suspect the species of the infective pathogen. Advanced diagnostic methods that are quite specific, fast, sensitive and easy to perform include a rapid antigen-capture dipstick test as well as the use of a fluorescent stain in order to identify the parasite (Fitri et al., 2022). Other than this, some of the methods that are majorly used in epidemiologic investigations and immunization trials include assays to identify malarial antigens and antibodies and polymerase chain reaction (PCR) DNA/RNA probe techniques. The various control measures that can be taken up for proper management of malaria include early detection of infection, rapid establishment of suitable therapy, and examining the clinical and parasitological response to therapy (Moody, 2002).

Therapy options related to malaria are quite complex as the pathogen can be found in the liver and blood of the infected host and thus various drugs are needed to eliminate each. Drugs that eradicate infections in the bloodstream are referred to as blood-stage schizonticides, whereas those that eradicate them in the liver are known as tissue schizonticides. Treatment options include combination therapy addressing both the erythrocytic and hepatic forms. The major antimalarials include hydroxychloroquine, chloroquine, primaquine and artemisinin-based combination therapy (ACT). Hydroxychloroquine and chloroquine disturb the erythrocytic stage by disrupting the hemoglobin metabolism of the pathogen and elevating the intracellular pH. Artemisinins remain therapeutically functional against all life cycle stages of malaria (Deroost et al., 2016; Bhattacharjee et al., 2023).

2.4 Beneficial role of protozoa

2.4.1 Role of human protozoa in molecular therapy (symbiosis therapy)

Symbiosis with its literal meaning as "living together" is defined as an organism that survives in a group and provides each

other collective benefits. Hence, approach around symbiosis is suggested as a disease therapy model as it is assumed that protozoa that inhabit naturally in human tissues can be genetically altered for the production and transfer of curative proteins. One of the examples for such kind of therapy model is *Leishmania* that has been genetically modified for conditional auxotrophy and indicated no disease-causing signs in non-human primate and mouse model-based clinical examinations. Numerous protozoan species have been transfected with a plethora of genes and have been observed producing active foreign proteins quite efficiently hence contributing their positive attribute on the medicinal front (Vaccaro, 2000).

2.4.2 Protozoa in wastewater treatment

Wastewater treatment is considered a pivotal method in the context of the ever-increasing human population. Wastewater processing facilities are intended to manage higher activity and density measures of those microorganisms that actively participate in various purification methods. With context to this, protozoa are considered to be responsible for repairing the nature of the effluent as well as controlling the bacterial population by predation and hence are taken as one of the most pivotal parts in the man-made system environment and play a crucial role in wastewater purification. Investigations performed on ciliates considering acute toxicity of contaminants suggested protozoans as potential bio-indicators for measuring water toxicity contaminated by various metal doses (Madoni, 2011). One of the research investigations examined the bacterivorous behaviour of ciliates that were isolated from artificial wetlands using the root zone method of wastewater treatment. Examples of ciliates that were seen to graze on fluorescently labelled bacteria, most notably *Escherichia coli* include *Paramecium* spp. oxytrichids, *Halteria* and scuticociliates suggesting their positive role in wastewater treatment (Decamp and Warren, 1998). Additionally, it has been established that *Uronema marinum* is an indicator of eutrophication and situations that are both oxygen-poor and rich in organic matter, while algivorous ciliates like *Tintinnopsis baltica* and *Favella ehrenbergii* are indicators of low nutrient levels and bacterivorous/detritivorous environments (Ravindran et al., 2023).

3 Fungi

The realm of fungus is made up of a wide variety of fungal species that are highly diverse and distinct from one another. There are numerous dangerous fungal species throughout this enormous planet. The majority of them are infections that affect plants, but some of them can be fatal to humans, animals, and other living things. The fields of medical mycology and microbiology have integrated the study of these hyphal infections and are no longer considered to be distinct fields. Fungal diseases are extremely important for public well-being: More than 14 billion global cases are reported of which over 1.5 million annual deaths are related to fungal infections (Casadevall, 2007). A fungal infection primarily affects immunocompetent, healthy individuals. Complementarily, there has been a rise in the quantity of these patients experiencing catastrophic fungal infections.

3.1 Fungal infection dynamics

The body may be exposed to fungus through several methods such as inhaling them into the lung or taking them by eating or colonizing other surfaces. Such injuries of anatomic barriers allow for the colonization of certain susceptible persons with local diseases. For example, these main risk factors include HIV/AIDS, cancer, organ transplantation, inherited or acquired immune deficiencies and other immune-suppressive events. Invasive interventions, antibiotic usage, as well as pathogen sources, can give rise to nosocomial fungal infections that pose huge threats for hospitalized patients. Acute rapidly progressive fungal infection tends to be serious in terms of mortality, which is worse when compared with sub-acute and chronic infections. Therefore, the development of the disease is determined by the fungal virulence traits and the health of the host (Rokas, 2022).

3.2 Pathogenic mechanism in fungi by immune evasion mechanisms and other strategies

There are many ways by which the fungal pathogens have evolved strategies to dodge or manipulate the host immune system functioning. Mechanisms such as escaping into the inner spaces of certain cells like those in the immune system, silencing immune activation in addition to reprogramming of the host immune response (Maertens et al., 2002; Li et al., 2019).

3.2.1 Adherence to the extracellular matrix components

One of the most important steps in the course of infection is thought to be binding on the host tissue, which appears to be facilitated by the identification of certain Extracellular Matrix components, such as basement membrane laminin, tenascin, types I and IV collagens, and fibrinogen. Disseminated infection most likely develops metastatic foci of infection throughout the body after attachment to the sub-endothelial extracellular matrix (ECM). In *Candida albicans* germinating cells, a variety of cell-surface receptors (68, 62, and 60 kDa) with various affinities for laminin, fibrinogen, and C3d were discovered. A 72-kDa cell-wall receptor and a 37-kDa laminin-binding receptor were identified in *Aspergillus fumigatus*. A glycoprotein of 120 kDa known as BAD1, is the most well-studied adhesin in *Blastomyces dermatitidis*. It can facilitate the adherence of yeast cells to host cells or extracellular matrix proteins. Thus, pathogenic fungal parasites can destabilize the molecules of their host to obtain entry that aids in their survival and development.

3.2.2 Internalization into host

Entering eukaryotic cells allows harmful fungi to take advantage of the generally unsuitable environment as a place to proliferate or hide from the host immune system. *Candida albicans* can cause endothelial cells to undergo phagocytosis by polymerizing their microfilaments and microtubules. The actin cytoskeleton can undergo disorganization due to the direct influence of fungal products released by *Candida*, such as actin-rearranging Candida-secreted factor (ARCSF), acting on actin or associated proteins. This disorganization is subsequently accompanied by rearrangement of cellular actin, a decrease in membrane ruffling, and a decline in cell motility. Wasylnka

and Moore conducted an *in vitro* study exploring the uptake of two distinct strains of *Aspergillus fumigatus* in A549 lung epithelial cells, human umbilical vein endothelial cells (HUVEC), and J774 murine macrophages. The cytoskeleton of the host cell had to be rearranged for A549 to internalize the conidia. These results show that non-professional phagocytes can internalize a significant number of *Aspergillus fumigatus* conidia *in vitro*, and these cells may serve as repositories for immune cell evasion and host-disseminated immunity.

3.2.3 Regulation of pathogenicity via signaling mechanisms

The investigation of signal transduction pathways in pathogenic fungi holds particular significance due to their potential involvement in the control of pathogenicity. A wide range of cellular functions are regulated by cAMP signaling cascades in both pathogenic and non-pathogenic fungi. Adenylyl cyclase-dependent signaling pathways govern the development of certain phenotypes necessary for virulence in *Candida albicans* and *Candida fumigatus*. Strains of *Candida albicans* (CaCDC35) containing a mutation in the adenylyl cyclase gene are unable to go through the morphological transition from budding yeast to polarised form, which is required for the organism to reach its maximal virulence. By extending their membranes and eluding macrophage defenses, invasive *Candida albicans* yeasts form germ tubes inside phagolysosomes and swiftly invade macrophage late endosome and lysosome compartments. These pathways may aid in the pathogen's survival and pathogenicity. It appears that the uptake of *Candida* yeasts depends on protein kinase C (PKC) activity, needing intact actin filaments, and hence exhibiting phagocytosis-like features (Mendes-Giannini et al., 2005).

3.3 Fungal infection in humans

3.3.1 Aspergillosis

Aspergillus, a prevalent mold (a specific kind of fungus) that can flourish both indoors and outdoors is the major underlying cause of the disease aspergillosis. *Aspergillus*-related health issues are more likely to develop among individuals with compromised immune systems or respiratory disorders. Aspergillosis occurs in various forms, while some varieties are minor, others are extremely infectious (Barnes and Marr, 2006). About 8 different types of aspergillosis are described hereunder including:

a Chronic pulmonary aspergillosis: Occurs when lung cavities brought on by *Aspergillus* infections persist for 3 months or longer. There may also be the presence of aspergillomas (fungal balls) in the lungs (Denning et al., 2003; Graham and Nasir, 2019).
b Aspergilloma (Fungus Ball): It is caused by an *Aspergillus* ball that grows in the sinuses or lungs and often does not spread to the other body regions (Latgé, 1999).
c Allergic *Aspergillus* sinusitis: Occurs when *Aspergillus* stimulates the sinuses and produces signs of infection including headache, stuffiness and drainage without actually causing the infection (Panjabi and Shah, 2011).
d Allergic bronchopulmonary aspergillosis (ABPA): In this case, *Aspergillus* irritates the lungs and generates allergic symptoms like coughing and wheezing (Rajpopat et al., 2022).
e Invasive aspergillosis: It typically affects individuals with compromised immune systems or those individuals who have undergone organ or stem cell transplantation. This type of aspergillosis generally affects the lungs however, it can also spread to other body regions as well (Dagenais and Keller, 2009).
f Azole-Resistant *Aspergillus fumigatus*: Occurs when *A. fumigatus*, a type of *Aspergillus*, develops resistance to a number of medications used to treat it (Burks et al., 2021).
g Cutaneous (skin) aspergillosis: In this condition, *Aspergillus* penetrates the body through an opening in the skin and produces infection. If invasive aspergillosis originates from any other part of the body, like the lungs and spreads to the skin then it also results in cutaneous aspergillosis (van Burik et al., 1998).

3.3.1.1 Clinical features

Symptoms associated with various types of aspergillosis include cough, shortness of breath and wheezing (allergic bronchopulmonary aspergillosis), decreased smelling ability, stuffiness and headache (allergic *Aspergillus* sinusitis) (Roth and Schatz, 2013). Signs associated with aspergilloma involve coughing up blood and shortness of breath (Lee et al., 2004), whereas patients infected with chronic pulmonary aspergillosis display indications such as weight loss, shortness of breath, fatigue as well as coughing of blood in response to the infection (Denning et al., 2003; Schweer et al., 2014). Moreover, hemoptysis, dyspnea, persistent productive cough, and chest pain are also observed as common symptoms in chronic pulmonary aspergillosis patients. All forms of chronic pulmonary aspergillosis cause hemoptysis, which occasionally progresses to potentially fatal large hemoptysis (Hou et al., 2017). Apart from this, invasive aspergillosis can cause cavitation, nodules, gradual consolidation, and the formation of an abscess. This condition is typically seen in patients who have become only slightly immunocompromised (Raveendran and Lu, 2018).

3.3.1.2 Pathogenesis

About 90% of cases of invasive Aspergillosis are caused by the opportunistic pathogen *Aspergillus fumigatus*, which carries a notably high fatality rate. The primary mechanisms by which *A. fumigatus* becomes pathogenic to the host encompass direct infection with virulent factors of the pathogen, individual hypersensitivity reactions, or activation of the host's innate and adaptive immunity due to virulent factors when the pathogen colonizes the host. Over the past few decades, numerous factors have been associated with *A. fumigatus* virulence, including thermotolerance, cell wall composition and integrity, resistance to immune responses, production of toxins, nutrient acquisition during invasive growth, regulation of signaling pathways, and allergenic properties. This understanding notably advanced following the sequencing of the *A. fumigatus* AF293 strain's genome in 2005. Given its thermophilic nature, *A. fumigatus* can thrive at temperatures up to 75°C and proliferate particularly well at 55°C. This characteristic enables it to colonize and grow in decomposing or decaying organic matter and to infect mammalian host cells more effectively. Consequently, *A. fumigatus* pathogenicity is partially attributed to genes associated with thermotolerance, including thtA, cgrA, afpmt1, kre2/afmnt1, and hsp1/aspf12. Additionally, toxins produced by *A. fumigatus* can directly harm the

host and contribute to the fungus's pathogenesis, providing defense against predators. Many of these toxins are fungal secondary metabolites that can alter cell membranes, hinder cellular functions, or affect the synthesis of proteins, RNA, and DNA. Numerous toxins and associated genes of *A. fumigatus* have been studied, including the transcription factor laeA, diffusible toxic compounds from conidia, gliotoxin (gliP and gliZ), mitogillin (res/mitF/aspf1), hemolysin (aspHS), fumagillin, and verruculogen. Among these, gliotoxin stands out as the most potent toxin produced by *A. fumigatus*, capable of inhibiting monocyte apoptosis, T cell proliferation, cytotoxic T cell response, and macrophage phagocytosis (Gu et al., 2021).

3.3.1.3 Diagnosis and control

Histopathology, culture and direct microscopy ideally with optical brighteners are strongly suggested for diagnosis. Usage of serum and bronchoalveolar lavage galactomannan measurements is strongly advised as prominent diagnostic indicators for pulmonary invasive aspergillosis. Apart from this, it is highly advised that all clinically relevant *Aspergillus* isolates have their infection identified down to the species complex level. For the first line therapy of Pulmonary invasive aspergillosis, voriconazole and isavuconazole are recommended (Ullmann et al., 2018). Some of the control measures suggested for the prevention of aspergillosis include-.

a Protection from the surroundings by avoidance of tasks like gardening or yard work that require close contact with dirt or dust, usage of gloves when working with things like dung, moss or soil and cleaning skin injuries well with soap and water to lessen the possibility of developing a skin infection (Ullmann et al., 2018; Avery et al., 2019).
b Providing antifungal medication to individuals who have undergone stem cell or organ transplantation as they develop elevated risk towards invasive aspergillosis (Panackal et al., 2014).
c Blood testing may also prove to be useful for certain high-risk patients to identify invasive aspergillosis (Barton, 2013).

3.4 Beneficial role of fungi

3.4.1 Role of beneficial fungus in sustainable farming

Fungi is one of the most essential classes of microorganisms that could potentially be used in a variety of industries, including agriculture. Valuable fungi play a significant part in the long-term viability of agriculture (Watts et al., 2023). These fungi come in a variety of forms, including symbiotic and endophytic varieties. The potential of beneficial fungi could be used as an alternative to chemical pesticides for the management of plant pathogens, pests and weeds. Endophytic fungi, mushrooms, entomopathogenic fungi and dark septate fungi fall among the well-known groups of beneficial fungi. Certain beneficial fungi are also observed to be associated with the enhancement of plant growth. The beneficial effects of using fungi in agriculture encouraged the industries to use them to create biopesticides and biofertilizers which can further be used in place of synthetic chemicals and reduce the amount of chemical waste in the environment. Hence, fungi play a significant role in sustainable farming by gaining incorporation into integrated pest and disease management strategies (Lavado and Chiocchio, 2023).

3.4.2 Diagnostic approaches for fungal infections

There are various methods by which diagnosis of fungal pathogens can be established (Fang et al., 2023). Table 2 represents various strategies involved in the detection of infections caused by disease-causing fungi.

3.4.3 Antifungal therapies

Antifungal drugs interfere with various aspects of fungal cell biological function, thus preventing further growth of the pathogen (Sanguinetti et al., 2015). Figure 3 describe the types of Antifungal Agents acting on different sections of the cell causing prevention of fungal infection these drugs can be classified into several categories described as:

TABLE 2 Representing various advantages and disadvantages associated with diagnostic approaches used for detection of fungal infections.

Diagnostic approach	Advantages	Disadvantages
Clinical evaluation	Rapid and non-invasive	Subjective and may not be specific for fungal infection
Direct microscopy	Rapid and inexpensive	May not be sensitive enough to detect all fungal infections
Fungal culture	Gold standard for species identification and antifungal susceptibility testing	Time-consuming (may take several days to weeks)
Molecular diagnostics	Highly sensitive and specific	Expensive and requires specialized equipment
Serological tests	Non-invasive and can be used to diagnose systemic fungal infections	May not be sensitive enough to detect early infections or in immunocompromised patients
Radiological imaging	Can be used to diagnose invasive fungal infections affecting internal organs	May not be specific for fungal infection
Skin tests	Can be used to diagnose specific fungal infections and determine exposure	Not sensitive enough to diagnose active infection and may give false-positive results in patients with prior exposure
Endoscopy and biopsy	Definitive diagnosis of invasive fungal infection	Invasive and may carry risks
MALDI-TOF mass spectrometry	Rapid and accurate identification of fungal isolates	Requires specialized equipment and not widely available
Next-generation sequencing	Detailed genomic information about fungal pathogens, allowing for precise species identification and detection of antifungal resistance markers	Expensive and requires specialized equipment

a Azoles: Azoles like fluconazole and itraconazole inhibit the production of ergosterol, which is an important product for the cellular barrier in fungi. Primarily, these agents work well for superficial or systemic fungus infections.
b Echinocandins: Echinocandins include drugs like caspofungin and micafungin which hinder the creation of beta-1,3-D-Glucan, a component of the cell wall found in fungi. It has been proved that these types of antibiotics help in the treatment of almost all *candidal* and *aspergillus* species infections.
c Polyenes: The binding of the polyene antifungal agent, such as amphotericin to the ergosterol in the fungal cell wall leads to permeability disruption of the latter. Hence, preventing the cycle of the infection (Sanguinetti et al., 2015).

4 Worms

Worms are a diverse group of creatures that include helminths and nematodes, which can parasitize humans, animals, and plants. Over a billion people worldwide suffer from serious parasitic ailments, which have a devastating impact on morbidity and mortality. Worms have also proven to be useful as pivotal model structures for investigating host–parasite interactions, and targeted treatments in addition to the mechanisms associated with the disease. This section demonstrates the depth of understanding offered by the worm models such as *Schistosoma mansoni* and *Caenorhabditis elegans*, that provide insights into virulence factors, host immune responses, and pathways for intervention against both microbial pathogens and parasitic worms (Derakhshani et al., 2022).

4.1 Worms as esteemed model organisms

C. elegans has emerged as one of the most powerful model organisms in biology due to its rapid life cycle, ease of laboratory manipulation, simple anatomy, and wealth of genetic tools. A large number of studies have utilized *C. elegans* for pivotal discoveries related to developmental biology, neuroscience, ageing, and host-pathogen interactions. Figure 4 shows *Caenorhabditis elegans* as a model system for understanding human-associated maladies. Its transparent body enables unprecedented visual dissection of infection processes. Researchers have even crafted mini-biosuits to explore worm behavioural responses to pathogens. Among helminths, *S. mansoni* stands out given its vast clinical impact and toolbox of research resources. Chronic schistosomiasis afflicts over 200 million people, underscoring the need for better therapies. *S. mansoni* can readily infect mice, facilitating detailed molecular investigations. Importantly, its intricate multi-cellular structure provides closer similarity to human parasites than single-celled models. Cross-species omics studies have revealed deep conservation of pathways regulating worm development, making *S. mansoni* invaluable for the identification of new intervention targets.

4.2 Diverse immune strategies against worms

Worm pathogens interface in detail with host immunity in a multifaceted manner. Infection elicits inflammation mediated by cytokines like IL-5, eosinophilia, mucus manufacturing, and IgE. CD4 T cells orchestrate immune mobilization and granuloma formation to isolate worms. However, hosts face challenges in removing worms absolutely because of their elusive nature and immunomodulatory secreted products. For example, filarial nematodes secrete phosphorylcholine-containing glycoproteins that result in IL-10 and modify dendritic cells to facilitate patience. Tropical worms express novel chemokine binding proteins that selectively goal key chemokines to undermine the recruitment of herbal killer cells and T cells. The excretory-secretory antigen Omega-1 from *S. mansoni* eggs controls IL2 production by way of CD4 T cells to selectively hose down Th2 responses. These cases illustrate sophisticated co-evolutionary adaptations of worms to guide host immunity (Mouser et al., 2019).

4.3 Pathogenic mechanism in worms

4.3.1 Penetration in the host organism through parasitic secretions

The term "elastases" refers to the soluble serine proteases of *Schistosoma mansoni* cercariae. The transcripts of at least three of these serine proteases have been found in the precursor germ balls of the daughter sporocyst stage in the snail host, and they have been biochemically characterized from the secretions of the acetabular gland. The proteins of the stratum corneum and the epidermis, where the entering cercarial body forms a penetration tunnel, are most likely the substrate of the characterized serine proteases, and as the parasite penetrates the epidermis, its thick protective glycocalyx and cercarial tail are shed. Glycans are another component of cercarial secretions that is worthy of consideration as a virulence factor. The proteins found in soluble cercarial secretions have high levels of glycosylation. Mass spectrometry has discovered a large number of both N-and O-linked glycan structures, many of which are shared between the two stages of transmission, indicating a shared function by the stages entering and exiting the host. Therefore, as a component of the immune evasion system, glycans act as a covert barrier to divert antibodies from functional peptide epitopes that are susceptible to attack, drawing leucocytes away from the approaching larva to let them escape from the protective host machinery.

4.3.2 Parasite's tegument and immune evasion

A syncytial layer of cytoplasm connects the schistosome interface with the circulation, often known as the tegument, to cell bodies located beneath the muscle. Evasion of the host immune response primarily takes place on the surface of the tegument. The ability of a few exposed enzymes on the outer leaflet of the tegument plasma membrane to alter the parasite's immediate environment within the blood vessel has led to their being considered as possible virulence factors. The discovery that ATP-diphosphohydrolase functioned externally on the tegument raised the possibility that the enzyme may control the number of purine nucleotides surrounding the parasites, allowing them to evade the host's hemostasis by blocking ADP-induced platelet activation. It has also been observed that the external surface GPI-anchored ADP-ribosyl cyclase enzyme may catabolize extracellular NAD+ to stop host enzymes from using it to promote immunological responses. Additionally, tegumental alkaline phosphatase's production of adenosine from adenosine monophosphate has been proposed to have a localized immunosuppressive effect (Wilson, 2012).

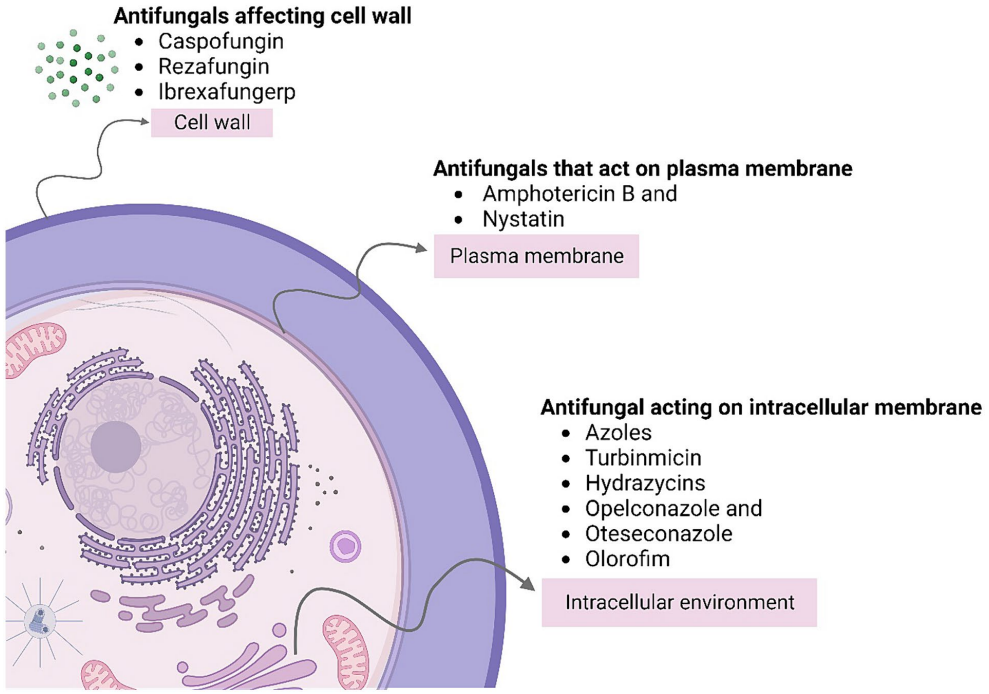

FIGURE 3
Describes the types of antifungal agents acting on different sections of the cell causing prevention of fungal infection.

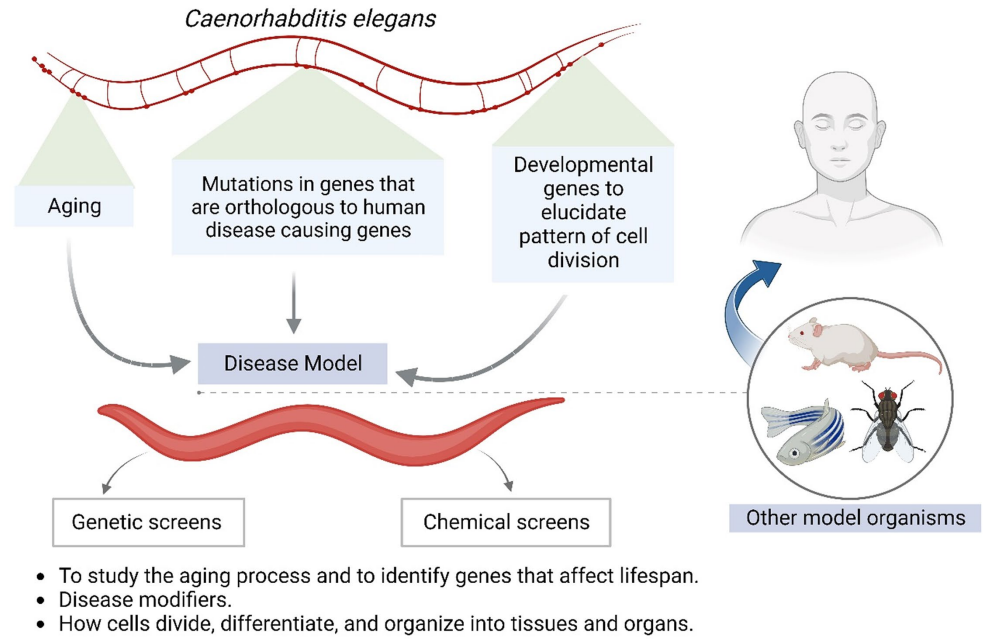

FIGURE 4
Shows *Caenorhabditis elegans* as a model system for understanding human associated maladies.

4.4 Diseases in humans

4.4.1 Fasciolosis (liver fluke)

Fasciolosis is an extremely infectious parasitic disease that infects humans as well as livestock and is caused by flat worms that belong to the genus *Fasciola*. *Fasciola hepatica* belongs to class trematoda and the infection caused by it is responsible for causing serious mortality and morbidity and is associated to elevated sensitivity to co-infections and decreased yield and potency. Consuming infected plants and vegetables is the primary way that humans acquire liver fluke infection. *F. hepatica* uses snails as intermediary hosts in its life cycle. When the pathogen finds appropriate environmental conditions, the

meracid undergoes the asexual phase of its life cycle after infecting the snail host. Eventually, it transforms into cercariae (Lalor et al., 2021).

4.4.1.1 Clinical features

There are two clinical phases of fascioliasis: acute and chronic. Signs and symptoms are contingent upon the phase, duration, and burden of worm infection. It is possible to detect eosinophil absence in the very early stages of acute illness. Few days later, an abrupt increase in the eosinophil count can be observed. When fascioliasis is highly suspected, it may be appropriate to repeat a blood test for eosinophil count 3 to 5 days later. Additional symptoms include lymphadenopathies, arthralgias, anorexia, weight loss, nausea, vomiting, coughing, diarrhea, and urticaria. When the parasite enters the bile ducts, the chronic phase starts. The majority of patients do not display any symptoms. When symptoms appear, they include urticaria, jaundice, nausea, vomiting, and recurring stomach discomfort in the right upper quadrant. All of these indicate biliary obstruction. At this stage, the liver has big, calcareous, dilated bile ducts that contain bile that is yellowish-brown in colour (Caravedo and Cabada, 2020).

4.4.1.2 Pathogenesis and host defense

Numerous factors influence the infectivity of metacercariae, including the ultimate host, parasite strain, climate, seasonal variations, snail species acting as hosts, and the developmental stage of snail larvae. As metacercariae represent the infective stage of *Fasciola* spp., the quantity of metacercariae ingested, the strain's isolation, and the type of host all play roles in the disease's progression. Shortly after ingestion by the mammalian host, metacercariae undergo excystment, and newly excysted juveniles (NEJs) promptly starts penetrating the host intestinal wall. In the stomach, elevated CO2 levels and a temperature of approximately 39°C trigger larval activation within the inner cyst. The upregulation of genes associated with cytoskeletal proteins such as talins and cell adhesion molecules like integrins and cadherins in metacercariae likely occurs due to their ability to sense environmental cues necessary for initiating the excystment process. NEJs release a plethora of virulence-associated compounds, including proteolytic enzymes and stage-specific peptidases, which degrade extracellular matrix (ECM) components and maintain tissue integrity. Five cathepsin cysteine peptidases, specifically cathepsin L3 (FhCL3) and cathepsin B peptidases (FhCB1, FhCB2, FhCB3, and FhCB9), exhibit high expression and subsequent excretion-secretion, facilitating rapid excystment of metacercariae and eventual invasion by NEJs into the host. Research indicates that these peptidases are essential for the parasite's pathogenicity (Lalor et al., 2021).

When comparing the pathogenesis to the infection level, the trauma-like stage produced by the juvenile flukes sifting through the liver parenchyma and intestines is largely associated. The symptoms of the acute stage may be mild or completely absent, but later on, they may develop into a complex chronic inflammatory condition. However, acute-stage infections are represented by active immune response towards the pathogenic antigens and are observed in the form of anaemia, fever, abdominal pain, hepatomegaly, transitional eosinophilia and increased levels of liver-associated enzymes With context to *F. hepatica* infection, Th2 response is observed majorly with short span of early Th1 response that is suggested to be functioning against the infection caused by the fluke. In case of animals having susceptibility to the parasite (for example sheep), a mixed reaction is observed considering Th1 and Th2 with IFNγ and IL-10 production.

4.4.1.3 Diagnosis and control

Human fasciolosis is relatively easy to diagnose in endemic places because the disease is sporadic there, but it might be difficult to diagnose in areas where the infection is rare. Diagnostic hallmarks observed in the patients include headache, fatigue, chills, sweats, abdominal pain as well as rashes. Apart from this, in order to detect hypodense liver nodules and lesion computerized topography (CT) is performed. In major duodenal papilla by utilizing endoscopic retrograde cholangiopancreatography live worms are visualized. Since no vaccine is available to provide protection from the infection, control on the individual level is suggested. Avoiding eating water plants and practicing good hygiene measures could be adopted to protect oneself from the infection (Morales et al., 2021).

4.5 Beneficial role of worms

4.5.1 Maintenance of soil fertility

All ecosystems benefit from the contribution of the soil biota to soil production and long-term viability. Earthworms (EWs) make up a significant amount of the biomass of macrofauna and are a major component of soil fauna communities in most habitats. Their activity is advantageous because it can improve the nutrient cycle of soil by quickly incorporating detritus into mineral soils. Apart from the effect of mixing, the creation of mucus resulting from water excretion in the stomachs of earthworms also amplifies the activity of other advantageous soil microbes. Furthermore, earthworms appear to quicken the process of soil organic matter turnover and mineralization. Through their direct and indirect effects on the microbial population, earthworms are also known to boost nitrogen mineralization. The potential for earthworms to aid in the stabilization and accumulation of soil organic matter in agricultural systems is shown by the increased transfer of organic carbon and nitrogen into soil aggregates (Bhadauria and Saxena, 2010). Some of the examples of earthworms that aid in the improvement and maintenance of soil fertility include *Hyperiodrilus africanus* and *Eudrilus eugeniae* (Tian and Badejo, 2001).

4.5.2 Vermicomposting

Earthworms and other microorganisms break down and stabilize organic waste through a process called vermicomposting. The organic waste substrates are broken up by the earthworms, which also significantly boosts microbial activity and accelerates mineralization rates. Since vermicompost is a stable, fine-granular organic matter, adding it to clay soil enhances airflow and loosens the soil. The mucus connected to the hydrophobic cast enhances water-holding capacity by absorbing water and preventing water logging. The plant can absorb the nutrients because of the organic carbon in vermicompost, which distributes the nutrients into the system gradually. Additional nutrients that are absent from artificial fertilizers are added to the soil when it is treated with vermicompost. Vermicomposting provides a way to recycle and utilize the tonnes of organic agricultural waste that farmers are burning to support our agricultural development in a more economical, ecologically friendly, and efficient way. Earthworms play a well-established function in the management of organic solid waste, and waste products can be processed using adapted technology to create vermicompost, an effective bioproduct. Vermicomposting is done with epigeic earthworms such as *Perionyx excavatus*, *Eisenia fetida*, *Lumbricus rubellus*, and *Eudrilus eugeniae*; however, native

species, such as *Perionyx excavatus*, have shown to be effective composting earthworms in tropical or sub-tropical environments (Garg et al., 2006; Ali et al., 2015).

5 Conclusion and future prospects

Our knowledge of the intricate relationships that exist between hosts and pathogens has been expanded as a result of host-pathogen investigations. The complex relationship between the immune system and invasive microbes has been a focus, offering insights into immune responses, infection processes, and pathogen evasion tactics. Hence, the information provided in this review can further help in providing ideas towards better exploration of this domain in a more targeted way. In summary, there are a number of promising avenues for future research in host-pathogen investigations, including personalized medicine, vaccine development, microbiome treatments, systems biology techniques, and combating antimicrobial resistance which in turn will help in enhancing global awareness for infectious threats.

Author contributions

RS: Visualization, Writing – original draft. JS: Visualization, Writing – review & editing. AK: Investigation, Writing – original draft. RP: Conceptualization, Funding acquisition, 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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Unveiling the orchestration: mycobacterial small RNAs as key mediators in host-pathogen interactions

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Small RNA (sRNA) molecules, a class of non-coding RNAs, have emerged as pivotal players in the regulation of gene expression and cellular processes. *Mycobacterium tuberculosis* and other pathogenic mycobacteria produce diverse small RNA species that modulate bacterial physiology and pathogenesis. Recent advances in RNA sequencing have enabled identification of novel small RNAs and characterization of their regulatory functions. This review discusses the multifaceted roles of bacterial small RNAs, covering their biogenesis, classification, and functional diversity. Small RNAs (sRNAs) play pivotal roles in orchestrating diverse cellular processes, ranging from gene silencing to epigenetic modifications, across a broad spectrum of organisms. While traditionally associated with eukaryotic systems, recent research has unveiled their presence and significance within bacterial domains as well. Unlike their eukaryotic counterparts, which primarily function within the context of RNA interference (RNAi) pathways, bacterial sRNAs predominantly act through base-pairing interactions with target mRNAs, leading to post-transcriptional regulation. This fundamental distinction underscores the necessity of elucidating the unique roles and regulatory mechanisms of bacterial sRNAs in bacterial adaptation and survival. By doing these myriad functions, they regulate bacterial growth, metabolism, virulence, and drug resistance. In *Mycobacterium tuberculosis*, apart from having various roles in the bacillus itself, small RNA molecules have emerged as key regulators of gene expression and mediators of host-pathogen interactions. Understanding sRNA regulatory networks in mycobacteria can drive our understanding of significant role they play in regulating virulence and adaptation to the host environment. Detailed functional characterization of Mtb sRNAs at the host-pathogen interface is required to fully elucidate the complex sRNA-mediated gene regulatory networks deployed by Mtb, to manipulate the host. A deeper understanding of this aspect could pave the development of novel diagnostic and therapeutic strategies for tuberculosis.

KEYWORDS

tuberculosis, *Mycobacterium tuberculosis*, small RNA, cis-encoded, trans-encoded, therapeutics

Introduction

Of all the infectious diseases known to mankind, tuberculosis is perhaps the most perplexing one. The discovery of the causative bacterium, *Mycobacterium tuberculosis* (Mtb) as its pathogenic agent by Sir Robert Koch in 1882, was heralded as a breakthrough in medicine. Nearly a century and half later, this infection continues to present a global challenge to healthcare (Comas et al., 2013). Moreover, evolution of this bacterium and excessive use of antibiotics has led to the emergence of Multi Drug Resistant (MDR) and Extensively Drug Resistant (XDR) strains. According to the WHO, TB caused an estimated 1.30 million deaths globally in 2022 (Global Tuberculosis Report, 2023). A lot of research has been done to understand the pathogenesis of Mtb and the mechanisms by which it can evade host defense. The survival and proliferation of Mtb in human macrophages is possible by a complex interplay of virulence factors. The expression of these virulence factors is regulated at transcriptional, post-transcriptional and translational stage. Amongst these, post-transcriptional regulation of gene expression by small RNAs has gained a lot of interest in the past two decades (Haning et al., 2014). Small RNAs (sRNAs) are tiny, versatile, non-coding RNAs which are 50–250 nucleotides long. They are involved in gene regulation by means of RNA interference and modification. sRNAs play a pivotal role in pathogenesis of several bacteria like *E. coli*, *B. subtilis*, and *Salmonella* (Waters and Storz, 2009). sRNAs were first identified in Mtb by northern blotting analysis in 2009 (Arnvig and Young, 2009). Early *in-vitro* research revealed that the expression of sRNAs like B55, F6 and ASpks is seen at low pH and high free fatty acid environment (Lovewell et al., 2016). These conditions mimic the activated macrophage environment and hence, it was postulated that sRNAs might help Mtb to survive the hostile macrophage environment (Vandal et al., 2009). Using high throughput sequencing techniques, many more sRNAs were identified (Arnvig et al., 2011; Ignatov et al., 2012; Pelly et al., 2012). Of late, sRNA Mcr11 has been shown to play a key role in the growth and central metabolism in Mtb (Girardin and McDonough, 2020). In this review, we have summarized the mechanisms of sRNA mediated regulation in Mtb and how they influence mycobacterial pathogenesis. We have also explored new avenues of sRNA secretion into the host that can pave way for novel theranostic approaches for TB that work at host-pathogen interface.

Bacterial small RNAs

Small regulatory RNAs (sRNAs) are considered to be major gene regulators in bacterial metabolism and survival (Schwenk and Arnvig, 2018). For regulation of gene expression, the majority of sRNAs base-pair with target mRNAs and modulate translation efficiency and mRNA stability (Waters and Storz, 2009; Storz et al., 2011). These sRNAs can be cis-acting if they are present at the same genetic locus or trans-acting if they are present on separate genetic locus. Trans-acting sRNAs are characterized by their involvement in base-pairing interactions with a limited number of RNA molecules and a small subset of protein-binding RNAs like the highly conserved *E. coli* 6S RNAs and CsrB, both of which modify protein activity, by binding to specific proteins (Schwenk and Arnvig, 2018).

Cis-encoded sRNAs

Cis-encoded sRNAs are small regulatory RNAs that are encoded on the opposite strand of their target mRNA and therefore, they are also called antisense sRNAs (or asRNAs). These asRNAs have the ability of extensive base-pairing in long regions with their target mRNA, often 75 nucleotides or more (Wagner et al., 2002; Brantl, 2007). Generally, cis-encoded sRNAs are expressed from plasmids, phages and transposons, but chromosomally encoded cis-encoded RNAs have also been discovered (Waters and Storz, 2009). These cis-encoded sRNAs maintain the copy number of the bacteriophages, plasmids, and other mobile genetic elements by preventing the synthesis of replication primer plasmid ColE1, RNA I and translation of transposons, as in the case of Tn10 pOUT RNA (Waters and Storz, 2009). Because of their perfect complementarity with their target mRNAs, true asRNAs, facilitate the recruitment of RNase III for degradation of target mRNA (Schwenk and Arnvig, 2018). Cis-encoded sRNAs are found to function as antitoxins in Type II systems where they code for proteases, which degrade toxin proteins, or they bind to toxin, not allowing it to act on target as in case of Type III A TA systems (Waters and Storz, 2009). Figure 1 illustrates the mechanism of action of cis-encoded sRNAs.

Trans-encoded sRNAs

Trans-encoded sRNAs are diffusible molecules of around 100 bp and they function in trans by binding to their target mRNA located at a distinct place away from their origin. Thus, the formation of such RNA duplexes is mediated by short and imperfect RNA interactions, usually 6–8 bp short 'seed sequence', which is extendable. Those sRNA sequence regions, which are involved in base-pairing and targeting multiple mRNAs, tend to be highly conserved than others, like GcvB sRNAs, of *Salmonella* and *E. coli*; these sRNAs have a conserved region which is G/U rich and which specifically binds to C/A rich region in its target mRNAs (Sharma et al., 2007). Due to this limited complementarity, these trans-encoded sRNAs can target multiple mRNAs (Gottesman and Storz, 2011). These are the widespread type of bacterial sRNAs and are found to be highly expressed in stresses like nutrient deficit, oxidative stress, etc. These sRNAs majorly show a regulatory role in the translation and stability of its target and have found analogous functional characteristics similar to eukaryotic microRNAs (Gottesman, 2005; Aiba, 2007).

In contrast to cis-encoded sRNAs, the absence of perfect complementarity in trans-encoded sRNAs necessitates their dependency on RNA chaperone, Hfq. Hfq facilitates chances of productive interaction between trans encoded RNA with their targets (Brantl, 2007; Waters and Storz, 2009; Vogel and Luisi, 2011). Gram-negative bacteria exclusively require Hfq which enhances base-pairing by binding to both trans-encoded sRNAs and target mRNA (Brennan and Link, 2007). Apart from assisting in function, this RNA binding protein is also involved in providing stability to sRNAs, in mRNA splicing and in decay too (Schwenk

Abbreviations: Mtb, Mycobacterium tuberculosis; TB, Tuberculosis; nc, Non-coding; DrrS, DosR regulated small RNA.

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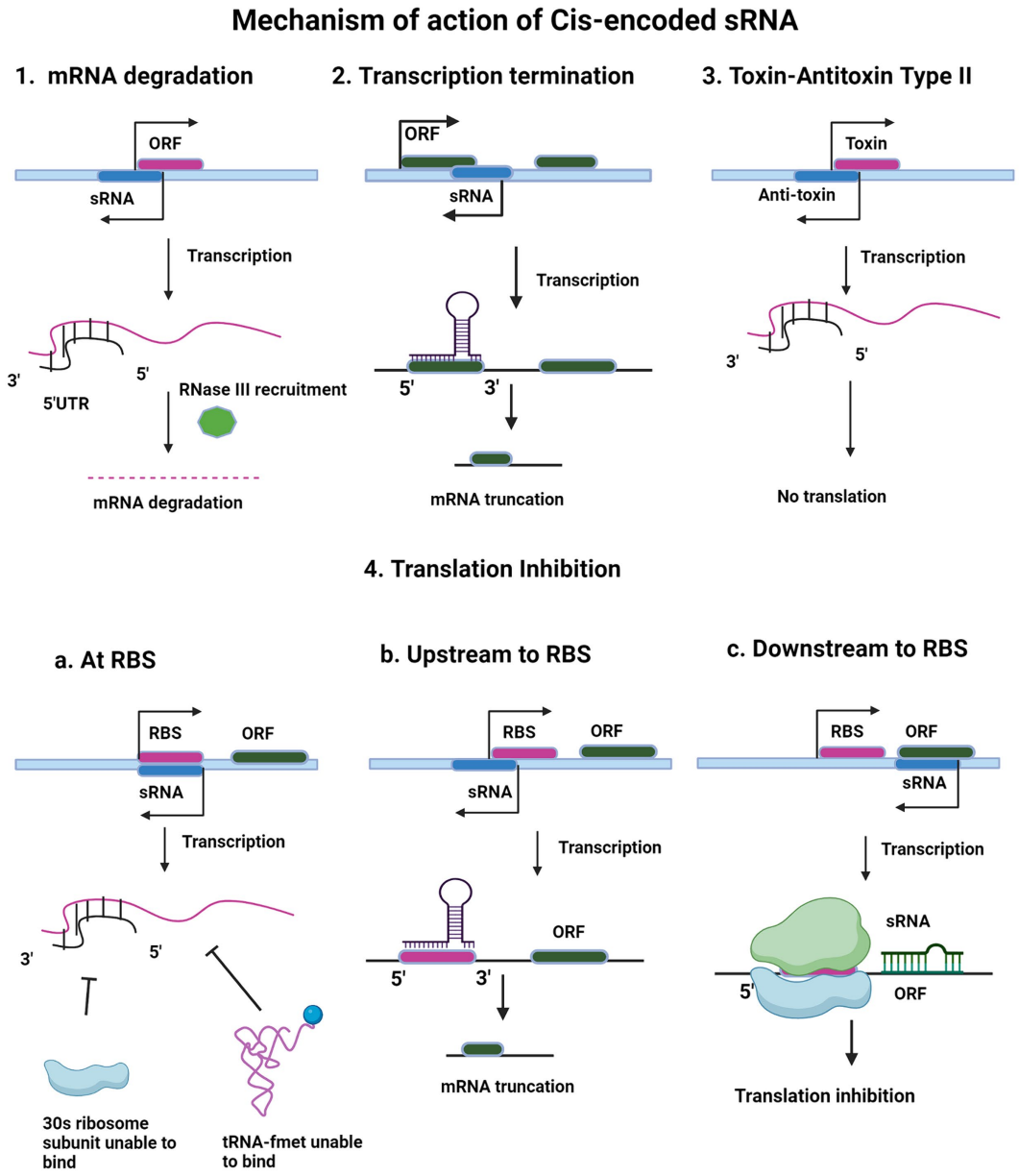

FIGURE 1
Regulatory mechanism of cis-encoded small RNA. The figure shows different mechanisms of action of cis-encoded sRNA like mRNA degradation (1), transcription termination (2), toxin-antitoxin Type II (3) and translation inhibition (4). In all the mechanisms, sRNA is depicted in blue color. Target ORF is depicted in pink color (1, 2, and 3). Upon intramolecular base pairing interaction of sRNA with its target, the different downstream pathways are shown in the schematic. After active transcription, base pairing occurs between the small RNA and complementary sequence in the 5′ untranslated region and initial segment of the ORF of the emergent mRNA. This RNA–RNA hybridization causes mRNA degradation (1) or premature termination of transcription (2). In toxin-antitoxin type II system (3), after transcription, the antitoxin transcript binds and pairs with the toxin transcript, inhibiting translation of the toxin gene transcript. In translation inhibition mechanism (4), cis-encoded sRNA blocks 30S ribosomal subunit and f-met tRNA binding. RBS sequence (purple) on target messenger RNA, obscuring 30S ribosomal subunit recognition, preventing translation initiation (4a). Open reading frame (ORF) is green. sRNA binds upstream of RBS on target mRNA, leading to truncated transcript (4b). Cis-encoded sRNA blocking of translation elongation is shown in 4c.

and Arnvig, 2018). Hfq requiring bacterial pathogens are found to show reduced virulence on its gene deletion (Chao and Vogel, 2010). These sRNAs are also found to have their internal terminator, uridine-rich end, a necessary element for forming the complex with Hfq (Morita et al., 2005; Ishikawa et al., 2012) There is a varying dependency of many species on Hfq. Bacterial species such as *Salmonella typhimurium* and *E. coli* have sRNAs that are highly dependent on this RNA chaperone, whereas, till now in gram-positive bacteria like *Mycobacterium tuberculosis* Hfq or its homologs have not been identified yet (Jousselin et al., 2009; Oliva et al., 2015), that is why the exact mechanism of how mycobacterial sRNAs interact with their target mRNA is still subject of investigation. Similarly, while *Helicobacter pylori* is a gram-negative bacterium, the identification of Hfq or its homologs in this species remains uncertain. Understanding the intricacies of sRNA-mediated regulation in these organisms necessitates exploring

alternative RNA chaperones or distinct regulatory mechanisms that may govern sRNA function in the absence of Hfq. Although, Cold Shock protein A (CspA), another RNA chaperone has its homolog present in Mtb (Arnvig et al., 2011; Caballero et al., 2018), and acts by denaturing the secondary structure of RNA (Jiang et al., 1997).

Structural aspects of base-pairing of trans-encoded sRNAs with their target mRNAs have been studied extensively in *Staphylococcus aureus*, where sRNAs SprD, RNAIII, and RsaE found their target mRNAs by utilizing C-rich regions present in the accessible site of the loop (Geissmann et al., 2009; Bohn et al., 2010; Chabelskaya et al., 2010). Some other sRNAs in *S. aureus* and other bacteria also utilize their C-rich region for recognizing their target mRNAs (Storz et al., 2011). Figure 2 illustrates various mechanisms deployed by trans-encoded sRNAs.

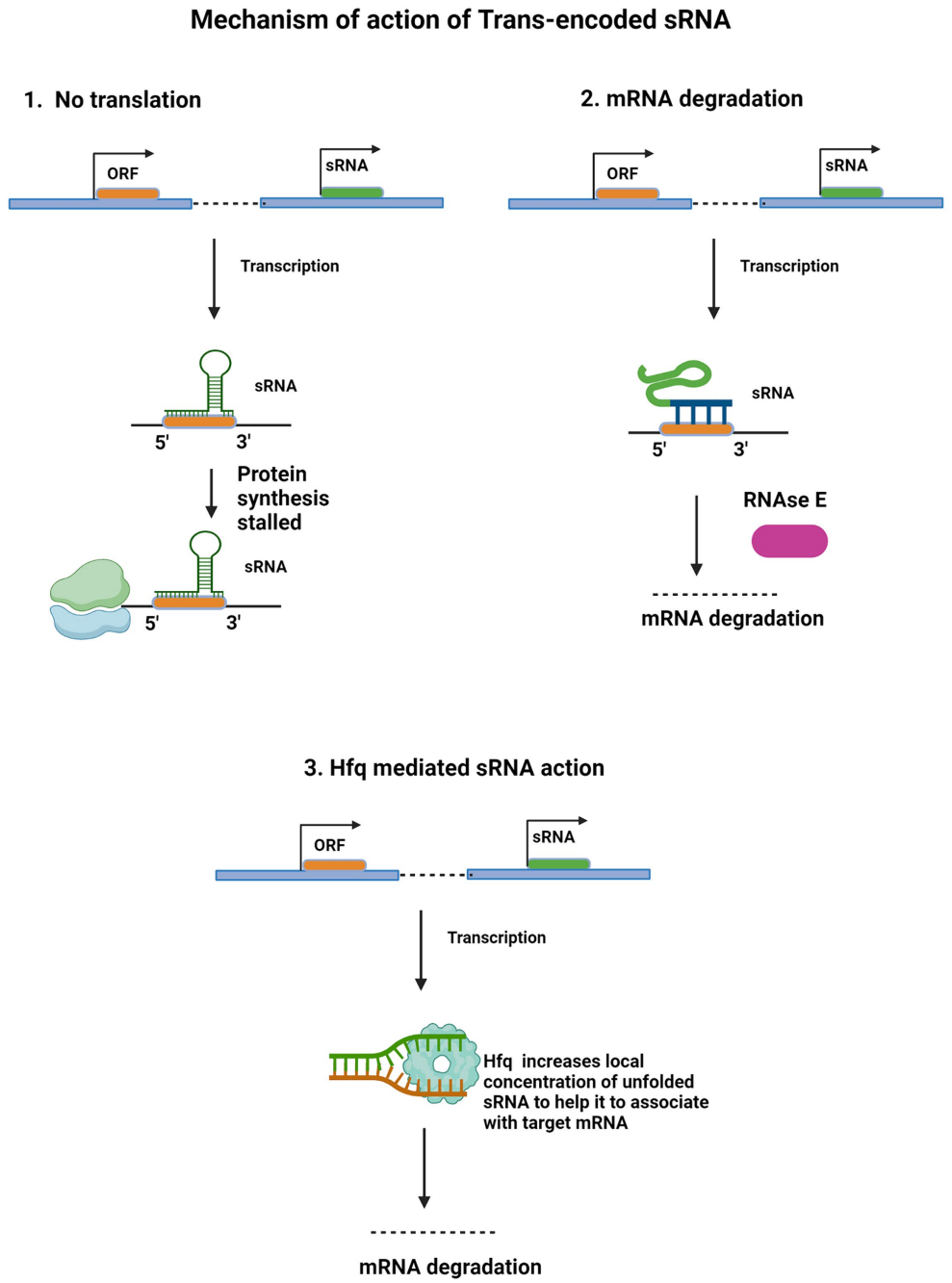

FIGURE 2
Regulatory mechanism of Trans-encoded sRNA. Trans-encoded sRNA (green) binds to complementary sequence in target mRNA open reading frame (ORF). The RNA duplex interacts with the 30S ribosomal subunit bound to the same mRNA molecule, obstructing translational elongation (1). After independent transcription, partial base pairing occurs between the sRNA and complementary mRNA sequence. This RNA duplex interacts with and recruits RNase E (purple), an endonuclease which cleaves target mRNA, leading to degradation (2). ORF of target mRNA (orange) and regulatory small RNA (green) are independently transcribed. Hfq protein (light blue) binds to and increases sRNA local concentration and availability to pair with target mRNA. By facilitating this base-pairing interaction, Hfq enables sRNA recruitment for mRNA degradation (3).

Mechanism of action of sRNA

sRNA base-pairing

sRNAs have some specific characteristics which are responsible for their diverse roles (Updegrove et al., 2015). sRNAs genes present within intergenic regions are mostly equipped with Rho-independent terminator containing a stem-region, followed by a poly-U stretch (Chen et al., 2002). This terminator is also found to be resistant to ribonuclease degradation (Ishikawa et al., 2012) and contains an Hfq-binding region (Updegrove et al., 2015).

Studies on cis-encoded sRNAs suggested that base-pairing requires various elements and interactions on several levels (Wagner et al., 2002; Brantl, 2007). The extensive complementary base-pairing between cis-encoded sRNAs and their target mRNAs does not occur immediately across the complementary length but starts with rapid and high-affinity base-pairing with the stem sequence located on the target itself. This initial interaction is referred to as "kissing" interaction (Storz et al., 2011). Following this, base-pairing may extend leading to RNA secondary structure rearrangements (Storz et al., 2011). A new study of the ibsC-SibC mRNA-sRNA duplex in *E. coli* shows the need for various structural elements and interactions at multiple steps (Han et al., 2010).

sRNA-mediated reduced mRNA stability

Reduction in mRNA stability is also one of the outcomes of sRNA mediated regulation. These sRNA-mRNA duplex formed after base-pairing between cis-or trans-encoded sRNAs and their target mRNAs cause these mRNA to get degraded (Gottesman and Storz, 2011). Trans-encoded sRNA-mRNA duplex is mostly degraded by the endoribonucleases such as RNase E or RNase III (Afonyushkin et al., 2005; Pfeiffer et al., 2009). Mtb has a homolog of both RNase E (present in *E. coli*) and RNase J (present in *B. subtilis*) (Even et al., 2005; Valerio et al., 2011; Durand et al., 2015). These two RNases are more specific for 5′ phosphorylated RNA, and there is a possibility that these RNases have the same function in Mtb as in *E. coli* and *B. subtilis, respectively,* (Mackie, 1998; Koslover et al., 2008; Li de la Sierra-Gallay et al., 2008; Mathy et al., 2010; Valerio et al., 2011). RNase E processes RNAs and causes their degradation (Lu et al., 2014). This endoribonuclease forms a complex called RNA degradosome to degrade mRNAs, in which its C-terminal region is bound with enzymes- polynucleotide phosphorylase (PNPase), enolase, and RNA helicase B (Morita et al., 2005; Carpousis, 2007). Through its C-terminal scaffold region, RNase E is also found forming ribonucleoprotein complexes with Hfq/sRNAs to recruit sRNAs at the target (Lu et al., 2014). Another endoribonuclease, RNase III is involved in regulatory actions of both cis-encoded and trans-encoded sRNAs (Brantl, 2007; Andrade et al., 2012), but the mechanism is still unknown (Lu et al., 2014).

The mechanism of trans-encoded sRNAs causing a reduction in target mRNA stability also involves several exoribonucleases, for instance, PNPase, a 3′-5′ exoribonuclease found to degrade single-stranded RNA and bind to Hfq (Mohanty et al., 2004; Morita et al., 2005). Apart from being involved in RNA degradosome mediated RNA decay, the PNPase also affects the stability of sRNAs such as MicA and RybB without requiring degradosome complex (Andrade and Arraiano, 2008) and is also responsible for turn-over of trans-encoded sRNA, but the mechanism behind it is still unclear (De Lay and Gottesman, 2011; Lu et al., 2014).

Inhibition of transcription and translation

Many sRNAs act by base-pairing in 5' UTR region, either at the ribosome binding site (RBS), where they prevent association of the 30S ribosome and fMet-tRNA (Holmqvist, 2012) or at a distant site upstream to RBS and halt translation, by making that site unavailable for ribosome binding (RBS) (Gottesman and Storz, 2011). This sRNA binding region can be at a distance of five codons from the open reading frame as in the case of *Salmonella* RybB sRNA binding to its target ompN mRNA (Bouvier et al., 2008) to 50 or more nucleotides upstream from the RBS (Sharma et al., 2007) where, they may adopt different mechanisms to block ribosome binding (Gottesman and Storz, 2011). In addition to this, RybB sRNA binds to mRNA coding sequence that prevents translation, but the first five codons of this coding sequence, also known as "five codon window", are required for efficient inhibition of translation.

In some cases, base-pairing at the downstream-regions of the ribosome binding site also occurs in which ribosome binding does not halt (Pfeiffer et al., 2009). This is exemplified by *Salmonella* MicC base-pairing with ompD mRNA at codons 23–26, without RBS (Pfeiffer et al., 2009). MicC sRNA promotes the RNase E activity for the ompD mRNA to get degraded by utilizing endo nucleolytic degradation of mRNA (Pfeiffer et al., 2009).

sRNAs can also cause termination of transcription of distal genes in an operon by recruiting the Rho-transcription factor, if it is bound at 5′ end, possibly by acting co-translationally to inhibit further translation, as found in ChiX sRNA of *Salmonella*, repressing the distal gene in chip cistron (Bossi et al., 2012).

sRNAs as an activator of transcription and translation

Small RNAs, especially, trans-encoded sRNAs can also function as mRNA translation activators, by preventing the development of inhibitory secondary structure, as its binding to the 5' UTR region of the target mRNA can stimulate conformational changes which could lead to the availability of RBS to the ribosome and therefore, activate translation (Morfeldt et al., 1995; Majdalani et al., 2005; Prévost et al., 2007; De Lay et al., 2013). This is well-exemplified by the action of the three sRNAs – DsrA, RprA, and ArcZ. Binding of any of these on the inhibiting structure of rpoS mRNA of *E. coli*, would unmask the initiation codon and Shine-Dalgarno sequence from the inhibitory structure of this mRNA, therefore, activate translation (Battesti et al., 2011).

Transcription activation has also been found in RydC sRNA which sequesters site for RNaseE action on cfa1 mRNA of *Salmonella* (De Lay et al., 2013). Other than this, sRNAs have been seen as an activator, because they block the Rho-factor binding site on rpoS mRNA and inhibit its Rho-dependent termination (De Lay et al., 2013). There is also a possibility of this anti-termination by sRNAs binding present in long 5′ leader sequences (Sedlyarova et al., 2016).

Hfq-mediated sRNA action

The RNA chaperone, Hfq, is a part of the conserved and ubiquitous RNA-binding Sm/Lsm (like-Sm) protein family, which has a role in mRNA degradation and splicing (Brennan and Link, 2007; De Lay et al., 2013). These are known to bind RNA with their ring or doughnut-like multimeric complex, wherein the bacterial Hfq protein is found as homomeric hexamer only (Gottesman and Storz, 2011; De Lay et al., 2013). Hfq performs remodeling of RNA structures without ATP hydrolysis. It facilitates the structural changes in non-coding RNA (Woodson et al., 2018).

Mechanism of interaction of Hfq with RNAs is still not fully clear, but analyses of the crystal structure of the Hfq-RNA complex found role of its structural surface in binding RNA molecules (De Lay et al., 2013). In *S. aureus*, the crystal of Hfq bound with the uridine-rich region of the RNA showed wounding of RNA on the Hfq proximal face in the central hollow region of its ring structure (Schumacher et al., 2002). Whereas in *E. coli*, Hfq crystal with the RNA bound on its Adenine-rich region revealed the distal face opposite to the proximal face (Link et al., 2009).

These trans-encoded sRNAs have a slight secondary intramolecular RNA binding at their 3'-UTR followed by the poly (U) region, which promotes Rho-independent termination of transcription. The initial contact of sRNAs can be at the proximal face itself (Storz et al., 2011; De Lay et al., 2013). There is also an Hfq binding domain adjacent to the poly (U) region (Storz et al., 2011). Other than this, some binding sites for Hfq are also present internally in sRNAs such as DsrA (Brescia et al., 2003), RyhB (Geissmann and Touati, 2004), RybB (Balbontín et al., 2010), and OxyS (Zhang et al., 2002). This internal site of binding for Hfq is further revealed in a study on SgrS (Ishikawa et al., 2012) which requires a U-rich region internally, adjacent to which, is a stem region for efficient Hfq binding (Ishikawa et al., 2012).

The distal face of Hfq has more-affinity for those mRNAs which characteristically have a binding region on ARN motif where, R and N refer to a purine and any base, respectively, as found in DsrA sRNA targeting rpoS mRNA (Soper and Woodson, 2008; Link et al., 2009). Studies found two Hfq binding sites on the rpoS mRNA leader, one in the proximity to the DsrA sRNA binding region and the other in upstream regions (Soper and Woodson, 2008), where the upstream regions having high-affinity for Hfq, enhance base pairing of this sRNA-mRNA duplex but proposed a hypothesis, that the second binding step following the initial base-pairing (De Lay et al., 2013).

Another site on Hfq termed lateral face and also the rim of the hexameric Hfq ring, showed the possibility of having a necessary role in stepwise combining with sRNAs which facilitate binding of mRNA and Hfq dissociation (Sauer et al., 2012). The current hypothesis for revealing the exact mechanism is that Hfq increases local RNA concentration, due to which Hfq needs to specifically target their sRNAs and mRNAs (De Lay et al., 2013). Further, model is proposed that as Hfq may enhance base pairing by performing remodeling of RNA structures as stated earlier (De Lay et al., 2013). The base-pairing begins initially between two hairpin loops or between a loop and a single-strand, which is followed by the base-pairing extension. Hfq facilitates base-pairing with both RNAs forming a ternary complex, which aids in rapid helix – nucleation (Woodson et al., 2018).

Mycobacterial sRNAs and mechanism behind their regulatory roles

The success of Mtb as a pathogen depends on its capability to adapt to the hostile environment of the macrophage showcasing stresses such as hypoxia, hydrolytic enzymes, antimicrobials, nitric oxide, nutrient limitation, oxidative stress, iron restriction, low pH, membrane stress and reactive oxygen species (Rohde et al., 2007). Within 20 min of phagosytosis by macrophage, Mtb shows differential expression of around 100 genes, which later increase to few hundreds (Rohde et al., 2007). Among Mtb sRNAs, MTS2823, ncRv12659, MrsI, DrrS, and Mcr7 are the most characterized ones (Arnvig et al., 2011; Houghton et al., 2013; Solans et al., 2014; Moores et al., 2017). The various stress conditions encountered by Mtb stimulates production of these sRNAs. Mtb sRNAs are mostly rich in GC content, making them structured to a highly appreciably structured (Schwenk and Arnvig, 2018). These RNAs do not possess usual intrinsic terminator, but possess another type of terminator, referred to as I-shaped terminators within them, which are devoid of uridine-rich 3′-end, unlike intrinsic terminators (Mitra et al., 2009). *In vitro* transcription and RNA seq studies showed insufficiency of I-shaped terminator, to cause transcription termination (Arnvig et al., 2011; Czyz et al., 2014). Because of which generation of 3′ termini occurs by processing in many Mtb sRNAs, unlike many sRNAs, that necessitates a poly-U tail for termination and dependency on Hfq RNA chaperone (Otaka et al., 2011). Table 1 summarizes regulatory roles of various sRNAs in the bacterium and the host.

Thus, the multifaceted roles of mycobacterial sRNAs in regulating gene expression underscore their significance in Mtb pathogenesis. Through their diverse mechanisms of action, including transcriptional regulation and mRNA degradation, these sRNAs contribute to the adaptation of Mtb to host stress conditions. Further research into the specific functions of characterized sRNAs, such as DrrS, Mcr7, and MTS2823, promises to unveil novel insights into Mtb-host interactions and potentially inform the development of targeted therapeutic strategies.

Regulatory roles of sRNA in Mtb

DrrS (DosR regulated small RNA)

DrrS (DosR regulated small RNA) was first identified in Mtb by RNA-seq (Lamichhane et al., 2013). DrrS is a trans-encoded sRNA, which is generated from precursor transcript, DrrS+ by rapid processing of the 3'end. This mature transcript is 108 nt in length and is highly structured, but its 3′ domain is less-structured which indicates its dependency on Rho-factor (Morgan et al., 1985). A high G:C ratio, a feature of Rho-binding site, appears in a small region of 26 nt only, downstream to 297 nt. Here, the longer transcript does not possess any intrinsic terminator in the initial 350 nucleotides. Collectively, the absence of intrinsic terminator structure, longer transcript DrrS+, and several 3′ termini presents within $DrrS^{+}$ suggests increasing possibility of Rho-dependent transcription termination of DrrS (Moores et al., 2017). Mycobacterial sRNA must go through 3′ processing (Otaka et al., 2011). This processing within DrrS+ is found to be done through the action of mostly both RNase E and RNase J, as Hfq homolog is

TABLE 1 List of different mycobacterial small RNAs and their role in Mtb and the host.

S. no.	sRNA	Structure	Role in Mtb	Role in host
1.	MTS1338	108 nt long and a stable secondary structure (Bychenko et al.). Has stem-loop structure	Promotes the expression of operons that cause growth defect in Mtb	• Helps Mtb to survive inside the host and enter a dormant state. • Induced in presence of NO derivatives, high IFN-γ levels, and low pH
2.	DrrS (DosR regulated small RNA)	• Has 5′ stem-loop structure • Processed from Drrs+(300 nt) to a stable DrrS (106 nt) (Morgan et al., 1985)	Regulates Rv1734 mRNA stability and gene regulation in response to to NO stress, hypoxia, and stationary phase (Park et al., 2003; Holmqvist and Wagner, 2017).	• Accumulates in high levels during chronic infection in mice (Arnvig and Young, 2012). • Pathogenicity of Mtb.
3.	MrsI (ncRv11846)	100 nt highly structured sRNA (Shell et al., 2015).	• Downregulates the expression of non-essential iron-containing proteins to conserve iron for essential bacterial functions. • Downregulates bfrA and fprA (Jacques et al., 2006; Oglesby-Sherrouse and Murphy, 2013).	Induced in the host under iron limiting conditions, oxidative and membrane stress (Richter and Backofen, 2012).
4.	F6	5′ end of the F6 sRNA is highly conserved.	• Regulates response to various stresses such as nutrient starvation, cold shock and oxygen depletion • hrcA gene (Dar et al., 2016; Holmqvist and Wagner, 2017).	Upregulates genes related to lipid metabolism Acetyl-CoA transferase and Acyl-CoA hydrogenase.
5.	Mcr7	• 350–400 nt • Has extensive folding and a 33-nucleotide long unstructured or free loop (DiChiara et al., 2010).	Targets 18 mRNA molecules including tatC and Rv2053c genes (Romilly et al., 2012; Solans et al., 2014).	By inhibiting the translation of tatC, it impacts the secretion of proteins involved in host-pathogen interactions, virulence, and immune evasion (Solans et al., 2014).
6.	AspkS	75 nt long, during oxidative stress an extended 200 nt AspKs transcript is induced (Arnvig and Young, 2012)	Downregulates pks7,pks8, pks12 and pks15	Found in TB patients' sera (Fu et al., 2018).
7.	Asdes	Extensive secondary structure with multiple hairpins and internal bulges (Schulze et al., 2016)	Downregulates DesA1	Found in TB patients' sera (Fu et al., 2018).
9.	As1726	Highly structured sRNA (27 nt)	Regulates tryptophanyl-tRNA synthetase trpS (Arnvig et al., 2011)	Found in TB patients' sera (Fu et al., 2018).
10.	As1890	Highly structured sRNA (36 nt)	Downregulates Rv1890	Found in TB patients' sera (Fu et al., 2018).
11.	MTS2823 (ncRv13661)	1st transcript: 300 nt located between genes Rv3661 and Rv3662c. 2nd transcript: ~250 nt appears during the stationary phase (Arnvig et al., 2011).	• Overexpression leads to down-regulation of growth related genes. • Targets mRNA Rv0115 (hddA), in GDP-L-fucose salvage pathway (Haning et al., 2014).	Found in infected lungs of mice, indicating its potential role in pathogenicity (Haning et al., 2014).
12.	MTS2048 (ncRv12659)	3′ region undergoes processing.	Differential expression of more than 50 genes (Houghton et al., 2013).	• 5′ region of ncRv12659 present during infection with Mtb (Houghton et al., 2013). • Potential biomarker for persisters (Betts et al., 2002; Voskuil et al., 2004; Rustad et al., 2008).

absent in Mtb. There is a perfect duplex between DrrS^{+} 3′ domain and Rv1734 mRNA, and it may be targeted by RNase III (Moores et al., 2017). It is found that the 5′ stem-loop of DrrS plays a significant role in stabilizing the DrrS, which is reflected by its long half-life. Also, from studies, it is strongly indicated that the 5′ phosphorylation state of recombinant variants of DrrS is found to be modified by RNase phosphohydrolase (RppH) homolog in *M. smegmatis*. Accumulation of DrrS sRNA is dependent on response regulator DosR. Since the sigma factor is expressed on DosR induction, this indicates that DrrS core promoter gets activated by the DosR by an unknown mechanism without requiring the DosR binding site (DBS). Research indicates that dosR gene activates the DrrS core promoter to a notable degree, but in certain tested conditions, both known DBS and the suspected DBS on the upstream region were found to reduce its expression (Moores et al., 2017). DosR is specifically induced during NO stress, hypoxic conditions and to a lesser extent in stationary phase and upregulates around 48 genes which help Mtb to adapt in the host (Park et al., 2003; Holmqvist and Wagner, 2017).

Mcr7

Mcr7 was found to be a greatly structured sRNA with extensive folding and a 33-nucleotide long unstructured or free loop, as predicted by RNAfold server. Mcr7 is a 350–400 nt, well-studied sRNA and is conserved in *M. tuberculosis* complex (DiChiara et al., 2010). High levels of Mcr7 are found in *M. tuberculosis* H37Rv through RNA-seq studies (Arnvig et al., 2011). It has been studied that Mcr7 targets 18 mRNA where 5′-end region shows complementarity. In some cases, Mcr7 interacts with its 33-nt loop (Romilly et al., 2012). Mcr7 basepairs with its unstructured part with its targets: tatC and Rv2053c genes (Solans et al., 2014). Mcr7 has complementary regions at its 5′-end for the tatC mRNA. These regions include the putativeribosome-binding site (RBS) and initial 6 codons of tatC mRNA. Binding of Mcr7 results in masking of RBS and halting of tatC mRNA translation. In Mtb, the tatC gene encodes a protein that is a transmembrane component of the TatABC secretory apparatus and is needed for translocation of secreted proteins from the cytoplasm to the extracellular environment with a twin-arginine or Arg-Arg (RR) motif present in their signal peptide, which are recognized by the TatC, before export through the TatA channel (Solans et al., 2014).

MTS2823 (ncRv13661)

MTS2823 is a highly abundant sRNA, around 300-nucleotide long transcript, expressed in exponential and stationary phase cultures (Haning et al., 2014). The Northern blot analysis showed one another form of MTS2823 transcript, around 250-nucleotides long during stationary phase (Haning et al., 2014). It is flanked by genes Rv3661 and Rv3662c. One of its target mRNA is Rv0115 (hddA), coding for D-alpha-D-heptose-7-phosphate kinase which has a role in the GDP-L-fucose salvage pathway. MTS2838 has also been found in infected lungs of mice, which indicates its role in pathogenicity (Haning et al., 2014).

The highest expression of MTS2823 sRNA takes place in stationary phase (approximately 10 fold more than in exponential phase) and adversely affects growth rate of Mtb (Arnvig et al., 2011; Ami et al., 2020). The genes associated with growth in exponential phases are down-regulated upon overexpression of MTS2823 (Arnvig et al., 2011). In Mtb during the exponential phase, 17% of the total non-rRNA are encoded from intergenic regions and referred to as sRNAs, whereas this amount increases to about 60% during the stationary phase, which is comparable to rRNAs, owing to the accumulation of MTS2823, a highly abundant sRNA. Higher than this amount, found in mice, having a chronic infection (Arnvig et al., 2011). Overexpression of its target mRNA Rv0115 causes downregulation of several genes by ≥2.5 fold and some of these targets are Rv3828c, hemD, mpt83, Rv0875, Rv3839, and ribH (Arnvig et al., 2011). It also decreases expression of methyl citrate synthase by around 15 fold (Haning et al., 2014). Overexpression of MTS2823 also leads to upregulation of two genes, Rv2035, encoding HspG activator (upto 3.2-fold) and Rv3229c, a fatty acyl desaturase DesA3 (up to 3.1-fold) (Arnvig et al., 2011; Haning et al., 2014). MTS2823 is also a functional homolog of 6S RNA, although its mechanism is still not clear (Arnvig et al., 2011; Haning et al., 2014). Downregulation of bacterial replication genes is mediated by transcriptional interference by 6S RNA expression, where RNA polymerase associated with principal sigma factor performed the transcription (Trotochaud and Wassarman, 2005). Genes downregulated because of overexpression of MTS2823 are prpC, ppdK, glcB, prpD, lrpG, icl, Rv0465c, Rv1128c, and Rv1126c, where prpC and prpD are methyl citrate genes and lrpG is their regulator, and others are their neighboring genes. Those which are downregulated by 2–2.5-fold are Rv1132, Rv1627c, polA, Rv3075c, acn, pckA, gka2, Rv0843, citA, acs and ltp3. The downregulation of methyl citrate gene prpC is because of the downregulation of several sigma factors, caused by overexpression of MTS2823 (Manganelli et al., 2001; Sun et al., 2004; Arnvig et al., 2011). The overexpression of the MTS2823 downregulated VapC toxin, whereas antitoxin partners did not seem to be affected.

Through STRING database analysis, an extended network of methyl citrate genes was seen getting down-regulated. The downregulation of specific genes such as prpC in particular and to an extent prpD indicates preferential targeting by MTS2823 to decrease either the utilization of propionyl-CoA and/or oxaloacetate or accumulation of toxic intermediates like methyl citrate (Arnvig et al., 2011). On the contrary to Hfq-dependent sRNAs, which degrades along with their target, MTS2823 appears to accumulate in stationary phase, and also during chronic tuberculosis infection in lungs of mice (Arnvig et al., 2011).

MrsI (ncRv11846)

Mycobacterial regulatory sRNA in iron (MrsI) or ncRv11846 is a trans-encoded, 100 nt long highly structured sRNA. Its transcription start site (TSS) is around 100 nt upstream of an unannotated gene, Rv1847 (Shell et al., 2015). It also has a predicted rho-independent terminator at its 3′ end (Czyz et al., 2014). The mycobacterial iron-dependent transcription factor, IdeR has a binding site near the TSS of MrsI in Mtb and *M. smegmatis* (Prakash et al., 2005) which could be the reason for the increased amount of this sRNA in iron-limiting conditions, and probably its involvement in iron-limiting response of bacteria (Gerrick et al., 2018). MrsI has bacterioferritin (bfrA), as its target mRNA, which is regulated by direct binding interaction. MrsI

has a short seed sequence of 6–8 nt which interact with the target mRNA and has 7-nt apical single-stranded loop. This was tested by transcriptional profiling in *M. smegmatis* during iron-limitation conditions, which identifies 20 MrsI regulated genes found in higher levels in *mrsI* deletion strain on comparing with wild-type ones. These 20 genes were assembled in 12 transcripts, and out of which, 8 were found to encode for nonessential proteins which play a role in iron metabolism which includes proteins as the bacterioferritin BfrA, the [NiFe] hydrogenase maturation factor HypF and ferredoxin reductase FprA (Gerrick et al., 2018).

Studies conducted on *M. smegmatis*, by fusing luciferase reporter gene to the MrsI promoter, found high levels induction in iron deprivation and not in other conditions such as oxidative stress and membrane stress in Mtb. bfrA and fprA are found to be regulated in Mtb as well. In Mtb, transcriptional profiling studies, combined with the CRISPR mediated knockdown of the sRNA, resulted in an abundance of 118 genes, and out of these, 106 were associated with iron deprivation, 12 with membrane stress and rest 5 were associated with oxidative stress (Gerrick et al., 2018).

MTS2048 (ncRv12659)

MTS2048 (ncRv12659) overlaps with the open reading frame of Rv2660c. It is the most up-regulated RNA in Mtb during starvation and is present at an adjacent site to the PhiRv2 prophage. The presence of only one Transcription start site (TSS) and no apparent terminators, led to the assumption that ncRv12659 is processed from the longer transcript (Houghton et al., 2013). Since TSS of MTS2048 is present within the PhiRv2 prophage, it may be concluded that only strains containing PhiRv2 exhibit sRNA expression (Houghton et al., 2013). High levels of MTS2048 cause growth defect in Mtb H37Rv. During starvation, an integrase promoter Rv2659c activates the sRNA promoter which indicates a repressive impact that triggers the phage lytic cycle (Houghton et al., 2013).

F6

5'end of sRNA F6 is highly conserved within *Mycobacterium* species (Arnvig and Young, 2012; Dar et al., 2016). It is highly induced during nutrient starvation, cold shock, oxygen depletion and repressed during heat shock conditions (Dar et al., 2016; Holmqvist and Wagner, 2017). Overexpression of F6 extremely slows down Mtb growth rate (Arnvig and Young, 2009) of Mtb. Comparative phenotypic and expression analysis between F6 WT, deletion and complemented Mtb strains revealed a relation between nutrient starvation and F6 expression (Houghton et al., 2013). During acid stress, the expression of F6 is induced by 2 fold and when Mtb is nutrient starved by static incubation for 96 h, F6 expression increases by >75 fold (Arnvig and Young, 2009). It specifically regulates expression of HrcA heat shock repressor in host. HrcA regulates expression of heat shock inducible chaperonins like Rv0440, Rv3418c and conserved hypothetical protein coding genes Rv0991c, Rv0990c (Stewart et al., 2003). Sigma factors regulate many genes involved in stress adaptations in Mtb during persistence state (Hu et al., 2000). F6 promoter contains a SigF promoter motif, which when overexpressed effects the growth rate of cells (Houghton et al., 2013). Anaerobiosis also induced expression of SigF followed by induced expression of sRNA F6 (Hu et al., 2000). When bacteria are given heat shock, SigF is downregulated.

In conclusion, exploring regulatory roles of small RNAs (sRNAs) in orchestrating gene expression dynamics contributes to our understanding of bacterial adaptation within the host environment. Through their diverse mechanisms of action, sRNAs such as DrrS, Mcr7, and MTS2823 play pivotal roles in fine-tuning the response of Mtb to various stress conditions encountered during infection. The identification of target mRNAs and the elucidation of sRNA-mediated regulatory networks provide valuable insights into the molecular mechanisms underlying Mtb pathogenesis. Continued research into the functional characterization of Mtb sRNAs promises to uncover novel regulatory pathways and potential therapeutic targets for combating tuberculosis. The next section discusses regulatory role of mycobacterial sRNAs in the host to facilitate the same.

Regulatory roles of mycobacterial sRNAs in the host

Many pathogenic bacteria use sRNA molecules to regulate cell cycle events, modulate their response in stressed conditions and thus virulence (Arnvig and Young, 2009, 2012; Haning et al., 2014). Apart from the role of sRNA in Mtb in the previous section, we will also describe role of some identified cis-encoded sRNA transcripts are ASpks, desA1, pks12, AS1726, and AS1890c in the host in this section (Betts et al., 2002; Arnvig et al., 2011). sRNA molecules regulate the expression of target mRNA. Research is being done to experimentally validate the associated roles of sRNAs in enhancing or repressing translation of mRNA targets (Betts et al., 2002). The following section will elaborate role of sRNAs identified in Mtb in host pathogenesis. Figure 3 shows diagrammatic representation of potential roles of regulatory mycobacterial small RNAs in the host.

DrrS

The presence of a stem loop structure at 5′ end makes DrrS a very stable sRNA, as this does not allow mycobacterial RppH homolog to bind and protects the 5′ end from RNAse activity. The levels of DrrS+ is highest during Mtb early stationary phase and DrrS108 accumulates during the late stationary phase at least up to 3 weeks (Moores et al., 2017) marking longevity in infection. The role of DrrS is dependent upon its processing. Both DrrS+ and DrrS108 have different regulons and hence different functions (Eruslanov et al., 2004). This understanding of stabilized regulation of mRNA can be applied to manipulate expression levels of different proteins in biological systems and to optimize immune system for vaccine development (Rohde et al., 2007). DrrS accumulates in high levels in mice during chronic infection, indicating its importance in achieving dormancy in the host (Arnvig and Young, 2012).

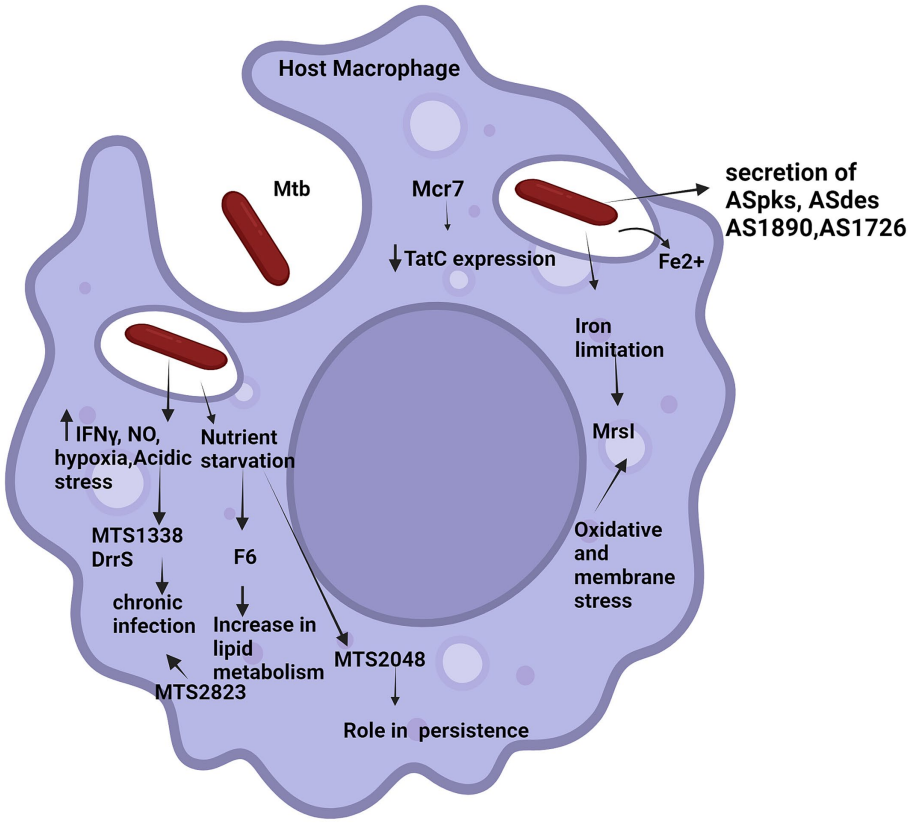

FIGURE 3
Potential roles of regulatory mycobacterial small RNAs in the host. The figure illustrates various external signals triggering small RNAs in host (various small RNAs have been represented by names). Solid arrows up/down indicate increase/decrease in the expression level of the molecules, respectively. Tapered arrows indicate downstream steps of different pathways.

MTS1338

Overexpression of trans-encoded MTS1338 helps Mtb to survive inside the host and attain dormancy which is followed by reactivation of the pathogen when the host immune system is down (Stewart et al., 2003). It was reported that mimicking host conditions like using nitric oxide derivatives, high IFN-γ levels and low pH induce expression of MTS1338 in mycobacterial cultures (Holmqvist and Wagner, 2017; Hör et al., 2018). Mycobacterial sRNA, regulatory proteins, and their cognate target proteins in Mtb work together to subvert the host immune system (Ignatov et al., 2015; Dutta and Srivastava, 2018). Lowering the pH enhanced the growth of the overexpression strain suggesting that overexpression of MTS1338 makes cells more resistant to acidic pH (Hu et al., 2000; Betts et al., 2002). *In vivo* studies with low doses of wild type Mtb caused relatively faster infection spread in I/St mice, which produce less IFN-γ than in B6 mice. It was found that 10 weeks post infection, MTS1338 expression in I/St mice was higher than in B6 mice (Ignatov et al., 2015; Dutta and Srivastava, 2018). Increased MTS1338 synthesis causes the metabolism to gradually shut down, which is one of strategies of Mtb for assisting its survival inside the host. Additionally, MTS1338 was expressed ten times more in IFN-γ activated macrophages than in control cells (Majorov et al., 2003). MTS1338 expression is largely triggered by NO, even in the absence of IFN-γ activation. This was discovered when MTS1338 expression could not be increased by IFN-γ stimulated macrophages, treated with L-NIL (NO synthase inhibitor) (Stewart et al., 2003). MTS1338-induced genes contributed to Mtb survival in oxygen- and energy-deficient environments. Down-regulated genes were found to be participating in translation, cell replication and biogenesis (Lovewell et al., 2016). These changes in transcriptional landscape induced by MTS1338 help the pathogen to reduce its metabolic activities and enter a dormant state.

MrsI (ncRv11846)

As discussed in the previous section, MrsI is expressed in *in vitro* cultures under iron-limiting conditions (Gerrick et al., 2018). It is a small independent transcript which downregulates its target regulon (bfrA/fprA) by directly binding to its 5' UTR region of mRNA and eventually degrading them. bfrA and fprA code for iron-containing proteins. When MrsI represses its regulons, it represses the expression of non-essential iron-containing proteins hence retaining iron for essential Mycobacterium functions. Rapid repression of bfrA is observed when Mtb is pre-exposed to oxidative and membrane stress before iron-deprivation. This may be an anticipatory response of Mtb to prepare itself for impending iron-deficiency. Mtb may use oxidative stress and membrane stress as the signals of warning that the host macrophage will soon get

deprived of iron, and thus it should start saving iron for its essential protein functions and enter an iron-sparing state. Thus, MrsI helps Mtb to anticipate iron deprived conditions, adapt, and survive in host macrophage.

MTS2048 (ncRv12659)

MTS2048 shows high levels of induction occurs during infection with PhiRv2 positive strain of Mtb, and this acts as a potential biomarker for identifying cells undergoing both starvation and hypoxia which later might turn into persisters (Betts et al., 2002; Voskuil et al., 2004; Rustad et al., 2008). It has also been observed that only a 5′ region of MTS2048 is present during infection with Mtb, which suggests transcription termination or 3′ end processing (Houghton et al., 2013). Differential expression of more than 50 genes was observed after overexpression of this sRNA, where PhiRv2 was found to be on top of the list (Houghton et al., 2013).

F6

F6 helps Mtb in intracellular survival within the host by upregulating genes coding for enzymes Acetyl-CoA transferase and Acyl-CoA hydrogenase which play an important role in lipid metabolism (Lovewell et al., 2016). Anaerobiosis conditions show induced F6 expression and Mtb transitions to persistent phase. The measured respiration rates show that ΔF6 strain is impaired while complement strain displays intermediate phenotype. This shows that F6 might have some role in reviving non replicating Mtb cultures hence intensifying host's later stages of infection (Lamichhane et al., 2013; Ignatov et al., 2015). As mice do not form granulomas, they do not generate a strong phenotype change. Thus, for this C3HeB/FeJ strain of mouse can be used as they have the ability to form lung granulomas (Houghton et al., 2013).

In summary, mycobacterial sRNAs play pivotal roles in modulating host immune responses and influencing the outcome of Mtb infection. By fine-tuning the expression of virulence factors and immune evasion strategies, these regulatory molecules shape the host-pathogen interaction landscape. The detection of mycobacterial sRNAs in TB patients underscores their clinical relevance and potential as diagnostic markers. Moving forward, elucidating the intricate interplay between sRNAs and host immunity holds promise for the development of innovative therapeutic interventions targeting Mtb infections.

Mycobacterial sRNAs detected in TB patients

ASpks

ASpks an antisense transcript that aligns with the mRNA, is a 75 nucleotide long transcript (Arnvig and Young, 2012), encoding duplicate identical ketosynthase domains within the pks12 gene in *M. tuberculosis*. The pks12 gene is involved in the synthesis of mannosyl-b-1-phosphomycoketide molecules, which are recognized as antigens by CD1-restricted T cells. ASpks has the potential to act as both a cis-encoded and trans-encoded sRNA, depending on the boundaries of the sRNA. ASpks shows significant complementarity to regions within pks7, pks8, and pks15, suggesting a potential regulatory role in the expression of these genes involved in polyketide synthesis (Arnvig and Young, 2009). ASpks was detected in the serum samples of active tuberculosis patients (Fu et al., 2018).

ASdes

ASdes can act as both a cis-encoded and trans-encoded sRNA, regulating the expression of desA1 and desA2 (Arnvig and Young, 2009). It shows significant complementarity to desA1 (Rv0824c), a fatty acid desaturase essential for the growth of Mtb and is upregulated during infection. ASdes also aligns with desA2 (Rv1094), another desaturase gene, indicating a potential regulatory role in lipid metabolism. It was detected in the serum of tuberculosis patients (Fu et al., 2018).

AS1890 and AS1726

AS1890 is a small RNA identified in *M. tuberculosis* with a size of 36 nucleotides (Arnvig and Young, 2009). AS1726 is another small RNA identified in *M. tuberculosis* with a size of 27 nucleotides (Arnvig and Young, 2009). These small RNAs (AS1726 and AS1890) were also found in the serum of patients, suggesting their presence and possible involvement in *Mycobacterium tuberculosis* infection (Fu et al., 2018).

To summarize, the identification of mycobacterial sRNAs in TB patients highlights their potential as diagnostic biomarkers for disease detection and monitoring. These sRNAs exhibit differential expression patterns in response to Mtb infection, suggesting their involvement in host-pathogen interactions. Leveraging these sRNAs as diagnostic tools may aid in early detection and treatment of TB, ultimately contributing to improved patient outcomes and disease management.

Mycobacterial sRNAs role in host immune response and therapeutic targeting

Mycobacterial small RNAs (sRNAs) play a multifaceted role in shaping the host immune response during infection. These small regulatory molecules play a crucial role in modulating the production of virulence factors that are critical for mycobacterial pathogenicity and survival in the host environment. Targeting particular genes and immune response-related pathways, sRNAs can precisely regulate immune signaling cascades, affecting the host's capacity to identify and eliminate the invasive pathogen. Furthermore, it has been shown that mycobacterial sRNAs modify the gene expression associated with immune evasion tactics, allowing the pathogen to evade host immune surveillance and establish persistent infections. Through their regulatory functions, sRNAs can orchestrate a delicate balance between pro-inflammatory and anti-inflammatory responses, shaping the

overall immune landscape during mycobacterial infection. Insights gained from studying the role of sRNAs in host immune responses not only deepen our understanding of mycobacterial infections but also hold potential for the development of novel therapeutic interventions (Haning et al., 2014). Some mycobacterial sRNAs may target host immune cell functions, such as macrophage activation or cytokine production, to subvert the host immune response and promote bacterial persistence (Coskun et al., 2021). The potential therapeutic targeting of mycobacterial small RNAs (sRNAs) presents a promising avenue for future medical interventions. One approach involves the development of antibiotics that specifically target sRNA-enabled virulence mechanisms, offering a novel way to disarm pathogens and overcome microbial resistance to traditional antimicrobials. Understanding the functions of small RNAs in modulating gene expression in mycobacteria opens up possibilities for RNA-based therapeutics that can disrupt pathogenic processes and enhance treatment outcomes. By harnessing the regulatory power of sRNAs, researchers seek to advance precision medicine approaches that target the molecular mechanisms driving mycobacterial infections, paving the way for more effective and tailored treatment options in the fight against these challenging diseases (Haning et al., 2014). Advancements in technologies such as CRISPR interference-based assays and locked nucleic acid (LNA) power inhibitors provide tools for studying and manipulating sRNAs, paving the way for the development of novel therapeutic strategies targeting mycobacterial sRNAs (Gerrick et al., 2018).

Conclusion

In conclusion, it can be said that small RNAs in Mtb form an intricate network of regulatory molecules that play a crucial role in bacterial adaptation, virulence, and pathogenesis. The ability of Mtb to survive and thrive within the hostile environment of the macrophage is facilitated by the differential expression of numerous small RNAs in response to various stress conditions such as hypoxia, nutrient limitation, oxidative stress, and increase in IFNγ. These small RNAs, including MTS2823, MTS2048, MrsI, DrrS, and Mcr7, have been identified as key players in the regulatory machinery of Mtb, responding to cues of the host and modulating gene expression for adaptation in the host. Small RNAs like MTS1338, MTS2823 and DrrS have role in chronic infection, while MTS2048 has a role in persistence (Betts et al., 2002; Voskuil et al., 2004; Rustad et al., 2008). The structural characteristics of Mtb small RNAs, such as their high GC content and unique terminator sequences, highlight their specialized mechanisms of action and processing. For instance, the DosR-regulated small RNA DrrS undergoes rapid processing at its 3′ end to generate a mature, highly structured transcript that is dependent on factors like Rho for termination. The intricate interplay between small RNAs, transcriptional regulators, and mRNA targets underscores the sophisticated regulatory landscape of Mtb. By targeting specific small RNAs involved in virulence or antibiotic resistance, researchers may uncover new avenues for combating drug-resistant strains of Mtb and improving treatment outcomes for tuberculosis patients. Moreover, the insights gained from studying small RNA networks in Mtb could have broader implications for unraveling similar regulatory mechanisms in other bacterial pathogens, paving the way for innovative approaches to combat infectious diseases.

Future directions

The study of small RNAs in Mtb holds significant promise for future implications in the field of tuberculosis research, diagnostics, and therapeutics. By delving deeper into the regulatory roles of small RNAs in Mtb and their interactions with host cells, researchers can uncover novel avenues. Small RNAs in Mtb that are crucial for virulence and pathogenesis could serve as potential targets for novel therapeutic interventions. By developing small molecule inhibitors or antisense oligonucleotides that specifically target these regulatory molecules, researchers may be able to disrupt essential pathways in Mtb that are necessary for survival in the host. By targeting key components of the small RNA machinery, researchers may discover innovative approaches to combat drug-resistant strains of Mtb and enhance the efficacy of existing antibiotics. Few sRNA (described in the previous section) have been detected in patients' sera. By identifying unique small RNA signatures associated with Mtb infection, researchers could develop sensitive and specific diagnostic tests for early detection of the disease, leading to improved patient outcomes and better disease management. The underexplored area is regulation of host genes by these secreted sRNA. By elucidating how small RNAs manipulate host gene expression, researchers may uncover new targets for host-directed therapies that enhance the immune response against Mtb infection. By profiling small RNA expression patterns in individual patients, clinicians may be able to tailor treatment regimens to target specific vulnerabilities in the bacterial population, leading to more effective and personalized therapies.

Emerging technologies in RNA sequencing, such as single-cell RNA sequencing (scRNA-seq) and long-read sequencing platforms, offer unprecedented opportunities to dissect the complex regulatory networks of small RNAs in Mtb. scRNA-seq can provide insights into the heterogeneity of bacterial populations within host tissues, shedding light on how small RNAs contribute to mycobacterial adaptation and persistence (Han et al., 2015). Long-read sequencing technologies enable the comprehensive characterization of small RNA structures and interactions, enhancing our understanding of sRNA-mediated gene regulation in Mtb (Han et al., 2015). Furthermore, the integration of RNA sequencing technologies into therapeutic development pipelines presents a promising avenue for novel treatment strategies targeting small RNAs in Mtb. By leveraging RNA sequencing data to identify key regulatory sRNAs involved in virulence, antibiotic resistance, and pathogenesis, researchers can design precision therapies that specifically target these molecules. Small molecule inhibitors or antisense oligonucleotides tailored to disrupt essential pathways in Mtb could lead to the development of innovative anti-TB treatments, particularly against drug-resistant strains. Additionally, the application of RNA sequencing in identifying unique small RNA signatures associated with Mtb infection in patients' sera holds potential for the development of sensitive and specific diagnostic tests for early disease detection, ultimately improving patient outcomes and disease management. By

harnessing the capabilities of RNA sequencing technologies in therapeutic research, researchers can unlock new avenues for combating tuberculosis effectively and advancing precision medicine approaches tailored to individual patients. This forward-looking approach not only deepens our understanding of small RNA networks in Mtb but also paves the way for transformative advancements in TB treatment and control.

Author contributions

RG: Conceptualization, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. IM: Investigation, Writing – original draft. DC: Data curation, Writing – review & editing.

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Conflict of interest

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Publisher's note

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The role of *Mycobacterium tuberculosis* exosomal miRNAs in host pathogen cross-talk as diagnostic and therapeutic biomarkers

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Tuberculosis (TB) is a global threat, affecting one-quarter of the world's population. The World Health Organization (WHO) reports that 6 million people die annually due to chronic illnesses, a statistic that includes TB-related deaths. This high mortality is attributed to factors such as the emergence of drug-resistant strains and the exceptional survival mechanisms of *Mycobacterium tuberculosis* (MTB). Recently, microRNAs (miRNAs) have garnered attention for their crucial role in TB pathogenesis, surpassing typical small RNAs (sRNA) in their ability to alter the host's immune response. For instance, miR-155, miR-125b, and miR-29a have been identified as key players in the immune response to MTB, particularly in modulating macrophages, T cells, and cytokine production. While sRNAs are restricted to within cells, exo-miRNAs are secreted from MTB-infected macrophages. These exo-miRNAs modify the function of surrounding cells to favor the bacterium, perpetuating the infection cycle. Another significant aspect is that the expression of these miRNAs affects specific genes and pathways involved in immune functions, suggesting their potential use in diagnosing TB and as therapeutic targets. This review compiles existing information on the immunomodulatory function of exosomal miRNAs from MTB, particularly focusing on disease progression and the scientific potential of this approach compared to existing diagnostic techniques. Thus, the aim of the study is to understand the role of exosomal miRNAs in TB and to explore their potential for developing novel diagnostic and therapeutic methods.

KEYWORDS

microRNAs, tuberculosis, diagnostics, therapeutics biomarkers, host pathogen interactions, immune response, bacterial exosomes, intercellular pathogen

Introduction

Tuberculosis (TB) is a severe bacterial infectious disease that poses a significant threat to global health and sanitation (Paul, 2024). In 2022, 10.6 million individuals worldwide were afflicted with TB. The disease can affect people of any age or gender, with an estimated 5.8 million cases among adult men, 3.5 million cases among adult women, and 1.3 million cases among children. These figures highlight the significant impact of TB on the global population. Additionally, an estimated 1.30 million fatalities from TB were reported in 2022. This figure is nearly back to 2019 levels, down from the peak estimates of 1.4 million in both 2020 and 2021 (World Health Organization, 2023).

Six million cases of TB were reported globally in 2021 (Bagcchi, 2023). However, it was observed in 2020 that TB has been steadily rising, especially among adolescents between the ages of 10 and 24 (Snow et al., 2020). TB is a highly transmissible respiratory illness caused by *Mycobacterium tuberculosis* (MTB) particles that infected individuals disseminate into the environment. Nevertheless, MTB cannot infect individuals unless their immune system is compromised (Moule and Cirillo, 2020).

MTB can infect nearly any part of the human body but primarily targets the lungs. It is important to distinguish between TB disease and MTB infection, as not all infections result in active TB (Karpinski, 2024). Furthermore, individuals with latent TB infection (LTBI) can be infected and show no symptoms, even though they typically test positive for TB through skin or blood tests. Proper TB tests are designed to detect latent infections, so a negative test result generally indicates the absence of infection? (Carranza et al., 2020).

Abbreviations: miRNA, Micro RNA; MTB, *Mycobacterium Tuberculosis*; WHO, World Health Organization; TB, Tuberculosis; LTBI, Latent Tuberculosis Infection; mRNA, Messenger RNA; DR, Drug-Resistant; MDR, Multiple Drug Resistant; ILV, Intraluminal Vesicle; ESCRT, Endosomal Sorting Complex Required for Transport; PAMP, Pathogen-Associated Molecular Pattern; DAMP, Damage Associated Molecular Pattern; PRR, Pattern Recognition Receptor; LAP, LC3 Associated Phagocytosis; 1,25D, 1,25-dihydroxy vitamin D; MVB, Multi Vesicular Bodies; RalA/B, GTPases Ral A and B Guanosine Triphosphatases; TSG101, Tumor Susceptibility Gene 101; SecA2, Specialized Secretion System A2; ESX-1, ESAT-6 Secretion System 1; MSCs, Mesenchymal Stem Cells; CCL5 C-C Motif Chemokine Ligand 5; iNOS, Inducible Nitric Oxide Synthase; TLR2/4, Toll-Like Receptor 2 and Toll-Like Receptor 4; MyD88, Myeloid Differentiation Primary Response 88; LPS, Lipopolysaccharide; CCL2, C-C Motif Chemokine Ligand 2; APCs, Antigen-Presenting Cells; MHC-I/II, Major Histocompatibility Complex class I and class II; DC, Dendritic Cell; THP-1 Tumor-associated Macrophage-derived Cell Line 1 ATM Ataxia Telangiectasia Mutated; AMPK, AMP-activated Protein Kinase; Ago-proteins, Argonaute proteins; siRNA, small interfering RNA; RNase III, Ribonuclease III; ADC, Adenocarcinoma; sRNA, Small RNA; Mcl-1, Myeloid cell leukemia sequence 1; STAT3, Signal Transducer and Activator of Transcription 3; ATB, Active Tuberculosis; SM, Sputum Smear; HC, Healthy Control; PPD, Purified Protein Derivative; IGRA, Interferon Gamma Release Assay tests; LAC, Lung Adenocarcinoma; circRNA, Circular RNA; LAM, Lipoarabinomannan; Ag85, Antigen 85.

Moreover, LTBI patients are among the few who can develop active TB infection (ATBI) at some point in their lives and play a significant role in spreading the disease (Ferluga et al., 2020).

MTB can persist by evading the host's immune system, mainly by altering its immune cells and the host's Micro RNA (miRNAs). These strains, such as MTB, reside in host macrophages, making them challenging to identify and eliminate (Bo et al., 2023). After transcription, miRNAs are crucial in regulating the expression of potential genes. They can bind to the 3′ untranslated regions of the messenger RNA (mRNA), causing either mRNA degradation or suppressing the translation process (Negrini et al., 2022).

The process of gene regulation by miRNAs is well-known and extensively discussed. Scholars have further revealed interactions between miRNAs and the immune system. Moreover, miRNAs are involved in the development of immune cells and influence their effectiveness, including macrophages, T cells, and B cells. Notably, three particular miRNAs—miR-155, miR-125b, and miR-21—have been proven to play roles in different immune responses to MTB (Zhao et al., 2019).

These miRNAs are critical in preserving fundamental signaling pathways and immune responses, significantly helping to determine the progression of the infection. They modulate the production of different inflammatory cytokines and play a significant role in regulating the activation and functions of macrophages, which are crucial for the clearance of TB and MTB (Singh et al., 2021).

Certain factors in the emergence of drug-resistant (DR) and multiple drug-resistant (MDR) TB complicate the diagnosis and treatment of TB. Therefore, developing new biomarkers with exceptional sensitivity and specificity is crucial for diagnosing TB (Jumat et al., 2023). Traditional approaches such as smears and MTB culture have limitations in determining the cause of TB. Some exosomal miRNAs are involved in TB development and can be distinguished from small RNAs that do not affect the host's immune response (Sharma et al., 2023).

For scientists studying the mechanisms of living organisms or the impact of different medications, it is equally attractive to investigate the actions of exosomal miRNAs released by MTB on macrophages. This manipulation allows the pathogen to counteract or evade the immune system, thereby maintaining its infection. Such miRNAs target genes and pathways linked to immune response processes and may be used for TB diagnosis and potential therapeutic target discovery (Carranza et al., 2021). Research should focus on identifying promising exosomal miRNA targets and developing innovative treatments for TB, highlighting their importance in managing the disease.

In this review article, the authors analyze the latest studies focused on the immunomodulatory properties of MTB exosomal miRNAs. They discuss how these miRNAs influence the host immune response and highlight their potential utility as biomarkers for tracking the progression and severity of MTB infection. The review provides an overview of the molecular mechanisms by which MTB exosomal miRNAs modulate immune functions and considers their implications for diagnostic and therapeutic strategies in TB management.

Understanding exosomes and miRNAs: formation and composition

Exosomes play a significant role in mediating cell-to-cell communication and are involved in the pathogenesis of MTB. These small membrane vesicles range in size from 30 to 150 nm in diameter and can be released into the extracellular matrix by virtually any type of cell (Dreyer and Baur, 2016; Li et al., 2020; Kang et al., 2021). Exosomes originate through endocytosis, forming small cup-shaped organelles known as early endosomes. These early endosomes facilitate the encapsulation of extracellular proteins, other molecules, and specific cell membrane receptors (Dreyer and Baur, 2016; Kalluri and LeBleu, 2020). During the transition from early endosomal to late endosomal identities, cargo molecules are concentrated on the early endosome's surface. Subsequent budding processes transport these cargo molecules into the intraluminal vesicles (ILVs) (Figure 1). These processes can be further organized into Endosomal Sorting Complex Required for Transport (ESCRT)-dependent and ESCRT-independent pathways, depending on the presence of ESCRTs in a cell (Eitan et al., 2016; Rayamajhi and Aryal, 2020; Gurunathan et al., 2021).

There are two primary biosynthetic miRNA pathways: the canonic and non-canonic signaling pathways (Sun et al., 2018). According to Hill and Tran, the canonic pathway is the principal route for miRNA biogenesis (Hill and Tran, 2022). RNA polymerase II transcription in the nucleus generates long primary miRNA with hairpin structures called pri-miRNA (Yu et al., 2016). Subsequently, the Drosha complex, which includes Drosha, Ribonuclease III (RNase III), double-stranded RNA–binding protein, DiGeorge syndrome critical region 8, and other partner proteins, cleaves the prior-miRNA into pre-miRNA with a stem-loop structure. In this stage, the pre-miRNA is processed to be transported in the cytoplasm. Once in the cytoplasm, the RNase III endonuclease Dicer processes the hairpin duplex into double-stranded miRNAs, producing a mature miRNA, the complementary strand, and the Argonaute proteins (Ago proteins), which are partial small interfering RNA (siRNA)-like molecules (Matsuyama and Suzuki, 2019; Kilikevicius et al., 2022). One of the two chains is chosen as the endogenous miRNA, whereas the second chain is generally cleaved (Riahi Rad et al., 2021) (Figure 1).

Following the MTB invasion of the respiratory organs, the immune cells, such as macrophages and dendritic cells, engulf the pathogen (Sia and Rengarajan, 2019). Macrophages and dendritic cells of the innate immune system detect MTB pathogen-associated molecular patterns (PAMP) through damage-associated molecular patterns (DAMP) or with the help of membrane surface pattern recognition receptors (PRRs) (Boom et al., 2021). Alveolar macrophages (MS) are particularly targeted in the initial stage of MTB invasion (Cohen et al., 2018). Recently, more research has focused on various types of receptors, such as scavenger receptors (Linares-Alcántara and Mendlovic, 2022), in connection with phagocytosis (Choudhuri et al., 2020). Therefore, to transport MTB within the cytoplasm and to form and translocate it into the phagosomes, immune cells must synthesize sphingomyelin on the cell surface (Niekamp et al., 2021). The phagosome pH drops during endosome-phagosome fusion, and then it fuses with lysosomes to create acidified phagolysosomes, which are essential for MTB suppression or elimination (Rai et al., 2022). This process, known as phagocytosis, involves LC3-associated phagocytosis (LAP) (Weiss and Schaible, 2015). After TB infection, macrophages activate bioactive 1,25-dihydroxy vitamin D (1,25D) via the vitamin D receptor, enhancing antimicrobial peptide synthesis (Cathelicidin Antimicrobial Peptide and β-defensin 2) and inflammatory proteins (IL-1β and IL-8), contributing to immunomodulation. MTB evades immune responses through strategies like apoptosis and modulation of innate immune cell responses (Wang et al., 2019). Multi Vesicular Bodies (MVBs) can fuse with lysosomes and, when subjected to lysosomal acid and proteolysis, become degraded. Moreover, MVBs can fuse with the plasma membrane and release the ILVs into the external milieu. Exosomes can also be formed by budding directly through the cytoplasmic membrane, assupported by previous studies (Eitan et al., 2016; Kalluri and LeBleu, 2020). When exosome secretion is inhibited, there is an increase of MVBs enhanced by lysosomal degradation (Eitan et al., 2016). The release and the fusion of exosomes with recipient cells membrane are closely regulated by the Ras superfamily. Several Rab proteins act as molecular signals in the movement of MVBs from one compartment to another. These proteins play a notable role in regulating vesicle transport, as highlighted in recent studies (Rayamajhi and Aryal, 2020; Gurunathan et al., 2021). Furthermore, the release of exosomes is facilitated by Ral A and B Guanosine Triphosphatases (RalA/B GTPases), which regulate effectors and lipid metabolism. These GTPases also control phospholipases D1 and D2, which are involved in maintaining MVB equilibrium and exosome cargo formation (Zago et al., 2019; Ghoroghi et al., 2021). The process described by Wickner & Rizo elucidates how Rab GTPase facilitates the assembly of membrane-bound soluble N-ethylmaleimide-sensitive factor attachment protein receptors into tetrameric coiled-coil complexes at both exosomal and receptor cell membranes (Wickner and Rizo, 2017), mediated by tethering proteins that colocalize the two membranes to allow them to come close (Borchers et al., 2021). In addition, exosome proteins include Cluster of Differentiation (CD) 63, CD81, CD9, flotillin, Alix, and Tumor Suceptibility Gene 101 (TSG101) (Figure 1). These proteins are implicated in the creation of exosomes, as noted by Gurunathan et al. (2021) in 2021. The exosomes biogenesis includes several pathways and electiveprocesses that determine their structural, transfer route, and cargo diversity acquired by cells from different sources. Overall, exosomes are crucial in cellular communication due to their ability to encapsulate diverse molecules through well-defined pathways. Their formation and release are complex processes regulated by various proteins and pathways, emphasizing their diverse roles in cellular communication and transport. Thus, their involvement in the immune response to MTB involves complex interactions between different cells and molecules, highlighting the pathogen's ability to evade immune defenses.

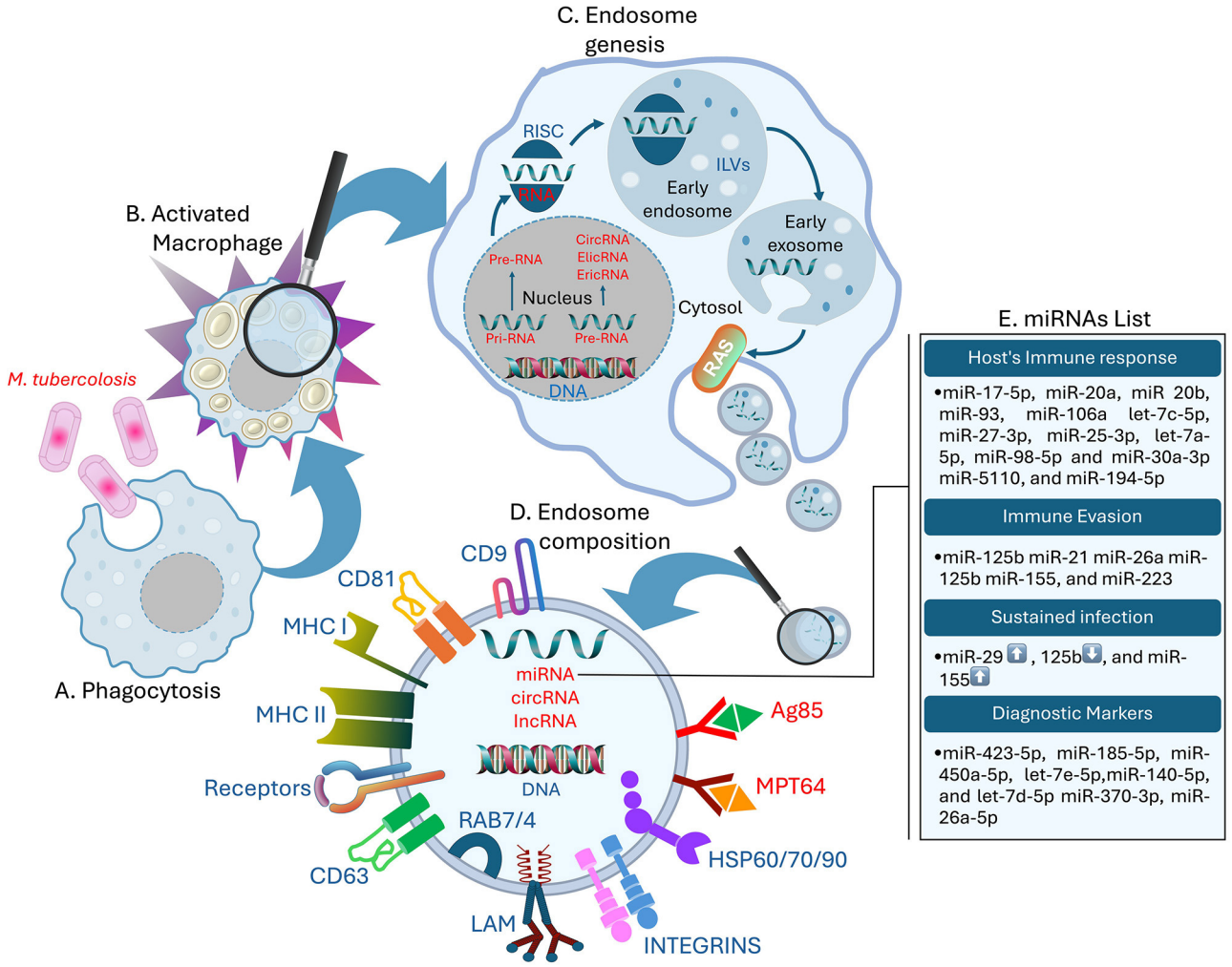

FIGURE 1
Role of exosomal miRNAs in the host's immune response. **(A)** MTB infecting the host macrophages, **(B)** activated Macrophage, **(C)** formation of the Drosha complex, ILVs, and release of exosomes from the host macrophages with bacterial non-coding RNAs (RNAs in red), **(D)** structure of the MTB exosome containing the necessary proteins. LAM, CD cluster, MHC-I/II, Receptors like PRRs, T-cell receptors, and Scavenger receptors; bacterial antigens like Antigen 85 and non-coding RNAs (RNAs in red), **(E)** list of exosomal miRNAs involved in host's immune response regulation, immune evasion, and up ↑ and down ↓ expression of sustained infection miRNA, and diagnostic markers.

The function of exosomes in MTB-infected hosts

Microvesicles contain diverse cargo, such as nucleic acids (e.g., miRNA, lncRNA, mRNA, DNA), proteins, lipids, and metabolites (Kugeratski et al., 2021). Exosomes, a subset of microvesicles, play a significant role in intercellular and intracellular signaling and communication between cells to regulate cellular processes and immunological defenses.

MTB relies on the Specialized Secretion System A2 (SecA2) and ESAT-6 Secretion System 1 (ESX-1) secretory systems for cell membrane degradation (Cheng and Schorey, 2019). Previous research by Tiwari et al. demonstrated the crucial role of exosomes in transferring genetic material, proteins, and other molecules within and between cells.

PRRs recognize these exosomes as PAMPs of the LAP pathway, triggering inflammasome activation (Tiwari et al., 2019). The cationic liposome driven by MTB-infected Mesenchymal Stem Cells (MSCs) compels macrophages to release pro-inflammatory cytokines, which include Tumor Necrosis Factor-alpha (TNF-α), C-C Motif Chemokine Ligand 5 (CCL5), and Inducible Nitric Oxide Synthase (iNOS). These factors escalate Toll-Like Receptor 2 and Toll-Like Receptor 4 (TLR2/4) and Myeloid Differentiation Primary Response 88 (MyD88) to induce inflammation and immune response (Liu et al., 2021). Consequently, the macrophage exosomes released by MTB-infected cells play a significant role in monocyte differentiation. This process involves the activation of specific molecules and proper pathways, resulting in the generation of functional macrophages (Singh et al., 2023). These exosomes are released following stimuli by lipopolysaccharide (LPS) and interferon-gamma (IFN-γ), most frequently by macrophages. These vesicles can bind soluble endoplasmic reticulum aminopeptidase-1; thus, this study was intended to address this interaction and the effects

it has on macrophages' phagocytosis and nitric oxide production (Goto et al., 2018).

Additionally, macrophages can engulf necroptotic exosomes, leading to the secretion of pro-inflammatory cytokines such as TNF-α, IL-6, and C-C Motif Chemokine Ligand 2 (CCL2); Chemokine (Shlomovitz et al., 2021). Exosomes secreted by Antigen-Presenting Cells (APCs) contain Major Histocompatibility Complex class I and class II (MHC-I/II), which present antigenic information to T lymphocytes, thereby triggering specific immune reactions (André et al., 2004; Ramachandra et al., 2010). Active T cells promote the production of exosomes from miR155-loaded dendritic cells (DCs), strengthening the activation of appropriate T cells (Okoye et al., 2014). Furthermore, T helper 1 (Th1) receiving let-7b-containing exosomes from Treg cellscounterbalance the excessive inflammatory response (Lindenbergh et al., 2019). Activated T lymphocytes transfer genetic material to DCs, aiding in the innate immune response to curb MTB infection (Torralba et al., 2018), while mitochondrial components play a crucial role in detecting and relocating DAMPs to various biological processes (Koenig and Buskiewicz-Koenig, 2022). Moreover, exosomes induce tumor-associated macrophage-derived Cell Line 1 (THP-1) autophagy (Sun et al., 2021b), and macrophage stimulation by exosomes secreted from MTB-infected neutrophils has also been observed (Alvarez-Jiménez et al., 2018). This stimulation provokes the formation of reactive oxygen species (ROS) and the generation of autophagy to eradicate MTB infection.

During infection, infected macrophages shed exosomes that deliver miR-18a into target cells; this specific miRNA interferes with autophagy, thereby promoting the resilience of MTB within the macrophages. A 2020 study conducted by Yuan et al. (2020) demonstrated the regulation of the Ataxia Telangiectasia Mutated-AMP-activated Protein Kinase (ATM-AMPK) autophagic pathway has been achieved. Macrophage-derived exosomes can also suppress the T-cell receptor expressed on CD4 + T cells and IL-2 production, as pointed out by Athman et al. in 2017 (Athman et al., 2017). Singh et al. (2011) found a reduction in IFN-γ levels with kinetics comparable to the reduction of CD64 or MHC-II expression in infected macrophages . Considering these findings, exosomes play a critical role in cellular communication and immune regulation, particularly in the context of MTB infection. They enhance immune responses and contribute to disease progression through various molecular pathways and interactions, making them potential targets for TB diagnosis and therapy.

Exosomal miRNAs: potential and synthesis

miRNAs are naturally occurring small RNA molecules of around 18–24 nucleotides that do not code for protein andare preserved throughout various evolutionary states (Iacomino, 2023). Moreover, miRNAs are pivotalin the modulation of several important biological processes, involving cell proliferation, differentiation, migration, apoptosis, and autophagy. They achieve this bytargeting gene mRNAs at the 3′-untranslated region (Song et al., 2019; Farina et al., 2020; Riahi Rad et al., 2021; Zhu et al., 2021).

miRNAs can coordinate multiple biological activities due to their complex regulation mechanisms. Their ability to remain constant throughout evolutionary stages emphasizes their evolutionary significance and adaptability in controlling gene expression. MiRNAs bind to target gene mRNAs at specific 3′-untranslated regions to alter genetic networks with remarkable precision. The complex biosynthesis of miRNAs involves transcription, cleavage by Drosha and Dicer, and the selection of functional miRNA strands, ensuring effective Ago protein-mediated post-transcriptional gene silencing.

The functions of exosomal miRNAs in the host

Exosomal miRNA from MTB-infected macrophages can be isolated using centrifugation techniques, making them suitable biomarkers for MTB infection. Zhang et al. (2019) found that exosomes from MTB-infected macrophages contain miR-20b-5p, while non-infected macrophage exosomes did not have this specific microRNA (Zhang et al., 2019). Recently, the same authors employed high-throughput sequencing to discover miRNAs in exosomes secreted by macrophages infected with *Mycobacterium bovis*.

The research revealed that the expression of 20 exosomal miRNAs unfolded increased in the samples of infected patients, while seven exosomal miRNAs decreased in the infected group compared to the non-infected group. Specifically, higher levels of let-7c-5p, miR-27-3p, miR-25-3p, let-7a-5p, miR-98-5p, and miR-30a-3p, and lower levels of miR-5110 and miR-194-5p were observed in the infection group (Zhan et al., 2022). In a 2016 study, Kumar et al. compared the quantitative alterations of several exosomal miRNAs in infected macrophages and the lung, spleen, and lymph nodes of MTB-infected mice to those in a control group. During MTB infection, miR-17-5p was found to be downregulated in both macrophages and mice. Other miRNAs from the same family, including miR-20a, miR-20b, miR-93, and miR-106a, were also downregulated in infected macrophages. The study revealed that miR-17 regulates the levels of Myeloid cell leukemia sequence 1 (Mcl-1) and its transcriptional activator Signal Transducer and Activator of Transcription 3 STAT3 in the context of MTB infection. By targeting Mcl-1 and STAT3, miR-17 plays a role in regulating autophagy. This finding highlights the importance of miRNAs, specifically miR-17-5p, in modulating autophagy and host responses during MTB infection (Kumar et al., 2016).

Exosomal miRNAs might be valuable for differentiating TB from other lung disorders. Wang et al. investigated the differences in exosomal miRNA in pulmonary adenocarcinoma (ADC), TB, and other diseases by evaluating their pleural distribution using qPCR. They found miR-205-5p, miR-429, miR-483-5p, miR-375, miR-200b-3p, and miR-200c-3p to be high in adenocarcinoma exosomes compared to tubercular and other pathology, while a downregulation of specific miRNAs, such as miR-3614-5p and miR-150-5p, was observed in malignant pleural. In addition, exosomes from TB were found to be laden with 148a-3p and 150-5p, which are lower compared to exosomes from non-cancerous tissues, showing contrasting results regarding the expression levels

of miR-451a (Wang et al., 2017). Exosome expression from LTBI, active tuberculosis infection (ATBI), and ADC patients' blood was evaluated by small RNA (sRNA) sequencing (Guio et al., 2022). Levels of miR-210-3p and miR-143-3p in serum exosomes from patients with LTBI decreased, while miR-20a-5p was upregulated in exosomes from patients' serum. Regarding ATBI serum exosomes, miR-23b, miR-17, and miR-181b-5p were downregulated, while miR-584 was upregulated. Finally, for ADC patients, 15 downregulated miRNAs were found, including miR-320a, miR-185-5p, miR-144-3p, let-7f-5p, and miR-199b-3p (Guio et al., 2022).

Exosomal miRNAs isolated from MTB-infected macrophages exhibit distinct profiles that hold promise as diagnostic biomarkers for TB. In light of this evidence, the differential expression of miRNAs in various clinical contexts underscores their potential utility in distinguishing TB from other lung disorders and monitoring disease progression. These findings advocate for further research into exosomal miRNAs to refine their diagnostic accuracy and clinical applicability in TB management.

miRNA-mediated manipulation by MTB

An evaluation score of exosomal miRNA found in MTB patients was determined, revealing their promise for rapid and non-invasive TB diagnosis. Research by Kaushik et al. (2021) showed that plasma exosomal miR-185-5p expression was significantly upregulated in the TB patient group compared to the healthy controls. Other authors also indicated that integrating miR-185-5p with other markers could significantly improve the diagnosis of TB.

A study conducted by Tu et al. (2019) identified higher levels of exosomal miR-423-5p in the plasma of TB patients. The TB diagnostic model achieved a score of 0. 908 with a 10-fold cross-validation mean prediction accuracy of 78%. The model demonstrated an accuracy of 18% and could differentiate between ATBI patients and healthy subjects, reflecting the findings of Kaushik et al. (2021). In the smear exosomes of LTBI patients, the levels of miR-450a-5p, let-7e-5p, miR-140-5p, and let-7d-5p were high. Additionally, in patients with LTBI, miR-370-3p and miR-26a-5p levels were higher than those in ATBI patients (Lyu et al., 2019). The results showed an escalating trend in the levels of these miRNAs among Healthy Control (HCs), LTB, and ATB patients. This distribution can be helpful in the recognition of different states of MTB infection (Lyu et al., 2019).

Alipoor et al. also found that the concentration of specific miRNAs in serum exosomes was significantly higher in patients with TB compared to healthy individuals. When these miRNAs were studied in combination with sputum smears, the diagnostic sensitivity for TB improved (Alipoor et al., 2019).

These research findings indicate that specific exosomal miRNAs hold significant potential for the rapid and non-invasive diagnosis of TB, demonstrating high diagnostic accuracy and the ability to differentiate between various stages of MTB infection. These findings highlight the importance of miRNA markers in improving TB diagnostics when combined with other methods, such as sputum smears, plasma analysis, and cross-validation models.

Role of exosomal miRNAs in immune regulation

The intrinsic properties of MTB enable it to survive through specific mechanisms. Following MTB infection, post-transcriptional regulation via miRNAs modulates target genes and the associated biological and immune processes. Indeed, miRNAs can inhibit translation or lead to mRNA degradation by specific mRNA target sites. This finding stems from the transcription mechanism of miRNA genes through RNA polymerase II and their post-transcriptional processing via endonucleases (Singh et al., 2022). miRNAs exhibit immunomodulatory functions for numerous immune cells, such as macrophages, T cells, and dendritic cells, playing a critical role in resistance against TB. This factor is significantly involved in the immune response to MTB in affected tissues. Ruiz-Tagle et al. (2020) provided experimental evidence on how the pathogen affects host cellular signaling through miRNAby analyzing dendritic maturation and its reference to the activation of T cells. The results confirm that miR-29 can decrease IFN-γ levels and change the T-cell response. Therefore, while IFN-γ is downregulated, T cells are actively triggered, and macrophages are reactivated. Through this manipulation, pathogens can remain dormant when the immune system is closing in and simultaneously reproduce (Ruiz-Tagle et al., 2020). Various miRNAs participate in the regulation of the immune response in MTB infection, managing the activities of macrophages, dendritic cells, and T cells. For instance, miR-125b, miR-155, and miR-223 significantly affect cytokines, cell cycle, and cell death, all related to the immune system (Yang et al., 2021).

Research conducted by Kozlov et al. (2023) has demonstrated that MiR-29 can influence or regulate IFN-γ, indicating that miRNAs play a role in the regulation of immune responses. Sarkar et al. showed that the downregulation of miR-125b can counteract the upregulation of TNF-α. Although TNF-α is an inflammatory agent beneficial for immune responses, excessive levels can inhibit immune reactions. MiR-125b targets the NF-κB pathway, which is significant for macrophages as it helps prevent prolonged inflammation, thereby aiding the eradication of MTB (Sarkar et al., 2024). In a recent study, Sun et al. explored the differences in infection levels when TNF-α is inhibited by miR-125b. This inhibition may occur when macrophages lose their ability to control and regulate the infection. These studies focus on understanding how miRNAs regulate host defense signaling pathways by targeting specific genes (Sun et al., 2021a).

Thus, the regulatory role of miRNAs, such as miR-29 and miR-125b, in modulating immune responses and host cellular signaling highlights their critical involvement in MTB survival and immune evasion, making them significant targets for understanding and potentially controlling TB infection. It is compelling to discuss the various processes by which MTB manipulates cellular elements. Among these processes, the regulation of miRNAs is notable, as MTB utilizes them to persist and proliferate. Understanding the pathogen's virulence involves examining how the host's miRNA expression, immune status, and cellular environment are affected.

Key miRNAs and their roles

According to a study by Alijani et al., there is a direct correlation between elevated levels of miR-155 and macrophage differentiation and functionality. This demonstrates how the bacteria might affect the immunity of the host. MTB can downregulate elements that promote anti-inflammatory processes, which are advantageous during an ongoing infection, by upregulating miR-155 (Alijani et al., 2023). Kulshrestha et al. discovered that miR-21 helps the bacteria grow by neutralizing genes that would otherwise cause the infected cells to die. Furthermore, MTB inhibits the miR-125b gene, which is essential for controlling the TNF-α cytokine, a major component of the body's fight against TB. Reduced TNF-α production results from lower levels of miR-125b, which lowers the TNF-α production, reducing the host's ability to combat the infection (Kulshrestha et al., 2019). Fu et al. (2020) highlighted the pathogen's ability to alter host immune responses for its survival by discussing how this manipulation of miRNAs, namely the inhibition of miR-125b, promotes the vulnerability of the host to MTB.

Kundu and Basu established that miR-26a plays a significant role in the immune response concerning MTB infection; it pertains to regulating cytokines belonging to the pro-inflammatory subdivision. This allows the pathogen to stay within the host because it avoids the aggression of the activated macrophages (Kundu and Basu, 2021).

According to Hu et al., miR-26a modulates a signaling pathway concerning several genes involved in immune response and inflammation mechanisms. Furthermore, miR-132 is linked with target genes that participate in the immunity reaction of the host organism. Discriminating from the cell aspect, it is noted that there is an upregulation of TB in cells, which, in turn, affects the production of other essential cytokines, such as TNF-α and IL-6, which are known to boost immunity. Therefore, this mechanism helps MTB escape elimination by the host's immune system and remains less toxic or capable of evoking an immune response (Hu et al., 2020). These findings are corroborated by Daniel et al. (2022) who noted that the host defense pathways in response to bacterial invasion could be affected by miR-132.

According to Wang et al., miR-29a can influence IFN-γ, hindering the immune system's ability to suppress MTB. Comparative analysis of the target miRNA focused on apoptosis pathways carried out by J. Wang et al. led to the discovery of the crucial role of miR-29a. This creates a cellular structure that enhances the growth of these bacteria. More importantly, IFN-γ mRNA is not the target of miR-29a; thus, the interaction between the two molecules is not the same. This is because IFN-γ plays a role in combating MTB as it stimulates the activation process of macrophages that are instrumental in the immune response (Wang et al., 2018). On the same note, miR-29a has also been observed to regulate the synthesis of another cytokine, IFN-γ. Specifically, miR-29a focuses on proteins implicated in apoptosis regulation, which will gradually pinpoint whether it is beneficial to hold cells alive soon after infection or otherwise eliminate them (Ruiz-Tagle et al., 2020).

Therefore, it is necessary to understand and determine the targets and functions of specific miRNA molecules, including miR-26a, miR-132, and miR-29a. Such understanding yields significant knowledge on how and through which MTB can evade several host cellular processes to increase survival (Crane et al., 2018).

The functions of these miRNAs in immune system regulation and their significance for TB are presented in Table 1. They play crucial roles in immunity, such as inflammation, T cell differentiation, and B cell activation. These processes are essential for the human body's ability to combat infection. These miRNAs have a dual role in modulating host defense and the pathogen's ability to promote infection. Understanding the mechanisms of action and molecular pathways of these miRNAs may provide potential treatment strategies for modulating these miRNAs to disrupt MTB's balance in managing TB.

Diagnostic potential of mirnas in TB

Challenges in current TB diagnostic methods

Currently, three widely-known TB diagnosis methods are the Mantoux tuberculin skin test, two types of IGRA tests, and sputum culture tests. However, these methods have demerits that cause their ineffectiveness and redundancy. The second important method of targeting the affected populace and identifying TB is the Mantoux tuberculin skin test, in which purified protein derivative (PPD) is inoculated into the skin. This test assists in determining the skin's reaction, in other words, whether a person has been in contact with TB bacteria. However, it cannot distinguish between the same individual's current and previous infections. Thus, tests like the QuantiFERON-TB Gold may further determine the immune response to TB antigens in the body, increasing the certainty level in diagnosing TB disease (Yang and Ge, 2018). It has been noted elsewhere that knowledge of how to manage DR-TB and MDR-TB is critical in eradicating this illness. Research suggests that exosomal miRNAs could be utilized to predict disease progression and diagnose either DR-TB or MDR-TB cases (Carranza et al., 2021).

Carranza et al. aimed to determine the baseline and 12-month post-treatment levels of exosomal miRNA in the serum of MDR-TB patients. Following therapy, they observed a drop in the blood levels of exosomal miR-328-3p, miR-20a-3p, and miR-195-5p. Conversely, let-7e-5p and miR-197-3p showed increased expression after therapy. The research also brought to light notable distinctions between the outcomes of those without cancer and those with cancer. Exosomal miRNAs have shown promise as biomarkers for DR-TB diagnosis and MDR-TB prediction. MDR-TB patients had lower serum levels of miR-197-3p and miR-223-3p than healthy controls, while their serum levels of let-7e-5p were higher. Certain miRNAs found in exosomes made from peripheral serum play a role in assessing the therapeutic impact of MDR-TB patients during phases of prolonged treatment. These results, which are related to variations in the serum of MDR-TB patients and healthy individuals, suggest that circulating miRNA can be utilized to assess TB strains' medication sensitivity or resistance (Carranza et al., 2021).

TABLE 1 miRNAs' roles in immune responses and their impact on TB.

miRNA	Immune system aspect	Function	Impact on TB response	Methodology	References
miR-155	Innate Immunity	Serves as an infection and activation marker, with higher expression in active TB	Indicates active TB, enhances immune response in macrophages	*In vitro*	(Alijani et al., 2023)
miR-125b	Innate Immunity	Modulates inflammatory response by targeting ROCK1	Promotes MTB survival in macrophages, impairing host defense	*In vivo*	(Sun et al., 2021a)
miR-223	Innate Immunity	Regulates myeloid cell function	Balances pathogen clearance with inflammation control, reducing tissue damage	*In vitro*	(Peng et al., 2022)
miR-29	Adaptive Immunity	Modulates inflammatory cytokine production	Overexpression can suppress IFN-γ, leading to inadequate immune response	*In vivo*	(Li et al., 2020)
miR-21	Adaptive Immunity	Modulates T cell differentiation and function	Ensures controlled and effective immune response, avoiding overactivity	*In vitro*	(Zhao et al., 2019)
miR-150	Adaptive Immunity	Involved in the regulation of immune responses, particularly B cell differentiation and antibody production	Influences B cell repertoire, potentially enhancing long-term immunity against MTB.	*In vivo*	(Sinigaglia et al., 2020)

As highlighted by Barry et al., miRNAs in infected patients assist in differentiating TB from other diseases. The evaluation of exosomal miRNAs reveals their high potential to impact TB-related conditions and behaviors. Consequently, these specific miRNA patterns may help diagnose and classify different types of TB. Additionally, exosomal miRNAs have been implicated in evaluating the effectiveness of anti-TB treatments, underscoring their potential to improve TB management (Barry et al., 2018).

Awareness is essential for TB eradication, as it helps design effective strategies to counteract the disease. However, there is currently insufficient literature on the effects of exosomal miRNAs on TB prognosis. The existing research on the influence of exosomes on TB prognosis is limited. Further research is needed to determine the potential of exosomal miRNAs in diagnosing TB and to expand our understanding of their roles in TB development. Identifying diagnostic and treatment biomarkers through exosomal miRNAs is essential for improving TB management.

miRNAs as biomarkers for TB diagnosis

Due to their TB specificity, miRNAs can be evaluated as biomarkers for the development of early diagnostic techniques (Olsson, 2024). Implementation of miRNAs for TB diagnosing in clinical can significantly improve treatment outcomes through proper and timely identification (Singh et al., 2021).

Three exosomal miRNAs (miR-484, miR-425, and miR-96) were assessed for their diagnostic utility in TB by Alipoor et al. The area under the curve (AUC) values of 0.72, 0.66, and 0.62 for serum exosomal miR-484, miR-425, and miR-96, respectively, were obtained using receiver operating characteristic (ROC) analysis. Furthermore, in TB-infected subjects, the levels of these three serum exosomal miRNAs were associated with smear TB positivity (Alipoor et al., 2019).

Lyu et al. (2019) compared the serum exosomal miRNA profiles of people with LTBI, ATBI, and healthy controls in a cross-sectional study. Four miRNAs were found to be elevated in the LTBI group: hsa-let-7e-5p, hsa-let-7d-5p, hsa-miR-450a-5p, and hsa-miR-140-5p. Furthermore, patients with ATBI had increased expression of five exosomal miRNAs: hsa-miR-1246, hsa-miR-2110, hsa-miR-370-3P, hsa-miR-28-3p, and hsa-miR-193b-5p. These results suggested that serum exosome miRNA patterns may be used to distinguish between LTBI and ATBI. The diagnostic efficacy of incorporating exosomal miRNA data into TB patients' electronic health records (EHRs) showed improved sensitivity and specificity compared to the tuberculin skin test and interferon-gamma release assays (Lyu et al., 2019).

Hu et al. assessed the mean ± SD of six distinct plasma exosomal microRNA types (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, and miR-486) in TB patients and found notable variations when compared to the control group. Including exosomal miRNAs and EHRs in the diagnostic model showed better diagnostic ability. Treatment success rates for patients with pulmonary TB and TB meningitis were reported to be 97%. Additionally, TB cases involving the organs frequently result in pleural effusion, which affects the pleura (Hu et al., 2020).

Kim et al. attempted to detect exosomal miRNAs across a variety of lung lesions, including lung adenocarcinoma, TB, and non-cancerous lung disorders, to differentiate TB from other lung diseases, such as lung cancer and pneumonia. Three miRNAs—miR-148a-3p, miR-451a, and miR-150-5p—were shown to be elevated in TB lesions compared to benign lung lesions using deep sequencing and qRT-PCR to quantify and compare miRNA expression (Kim et al., 2017).

Nine exosomal miRNAs in pleural effusion (PE) samples were found to be significantly different in the lung adenocarcinoma (LAC) group compared to the other two groups in a distinct study conducted by Wang et al. According to the authors, a few of these

TABLE 2 Some critical miRNAs' expression in TB, used as diagnostic markers.

miRNA	Expression	Samples extracted	References
miR-423-5p	High	Plasma of TB patients	(Tu et al., 2019)
miR-185-5p	Upregulated	Plasma exosome of TB patients	(Kaushik et al., 2021)
miR-450a-5p, let-7e-5p, miR-140-5p, and let-7d-5p	High	SM LTBI patient, serum exosomes	(Lyu et al., 2019)
iR-370-3p, miR-26a-5p	Higher expression	Serum of LTBI	(Lyu et al., 2019)

TABLE 3 List of miRNAs as potential biomarkers.

miRNAs	References
miR-4669-5p	Wang et al., 2023
miR-20b-5p	Zhang et al., 2019
miR-27-3p, let-7a-5p, let-7c-5p, miR-25-3p, miR-98 5p, miR-30a-3p, miR-194-5p, miR-5110	Zhan et al., 2022
miR-17-5p, miR-20b-5p	Tu et al., 2019
miR-1246, miR-2110, miR-28-3p, miR 193b-5p,	Lyu et al., 2019
miR-484, miR-425, miR-96	Alipoor et al., 2019
miR-205-5p, miR-200c-3p, miR-141-3p, miR-483-5p, miR-375	Wang et al., 2017
miR-33a-3p, miR-153-3, miR-373-5p, miR-3120-5p, miR-489-3p, miR-4669-5p	Zhang et al., 2022
miR-143-3p, miR-210-3p, miR 20a-5p and 23b, miR 17, miR 584	Guio et al., 2022

miRNAs were miR-205, miR-483, miR-375, miR-200c, miR-429, miR-200b, miR-200a, miR-203, and miR-141 (Wang et al., 2017).

The diagnostic potential of other miRNAs, including miR-146a and miR-125b, has also been examined. Numerous studies have demonstrated the impact of miRNAs on immunological responses and disease susceptibility in TB patients, indicating their potential as biomarkers for determining the presence and stage of the disease (Kundu and Basu, 2021).

Liu et al. established that miRNAs are specific and sensitive indicators for disease diagnosis. The authors can effectively diagnose diseases by using miR-155 and miR-29a, along with other specific miRNAs, as biomarkers (Liu et al., 2022).

According to the mentioned research, while discussing the diagnostic capacity of miRNA for TB, the emphasis should be on the specific types of miRNA. The miRNAs indicated in Table 2, including miR-423-5p, miR-185-5p, miR-26a-5p, miR-450a-5p, let-7e-5p, miR-140-5p, let-7d-5p, and miR-3700-3p, strongly affect the expression of the immunological system and the pathogenicity of TB.

Discussion

Exosomes are a promising tool for understanding the subtleties of the pathological process of TB, especially when released from cells infected with MTB. As for the structure and composition of exosomes, they have close relations to their function, modulating the host's immune response. Exosomes derived from MTB organelles called MVBs carry lipids, proteins, nucleic acids, and miRNA that are important for MTB to thrive, grow, and suppress the immune response. MTB has been known to use different proteins, such as ESAT-6 and Antigen 85 (Ag85), to control the host's immune response and survive within macrophage cells (Raposo and Stoorvogel, 2013). Some proteins present in MTB exosomes, such as lipoarabinomannan (LAM), may affect apoptosis and cytokine secretion. This interaction may enhance the immunological response to MTB, aligning with the pathogen's survival strategy. Interestingly, this adaptation also serves a critical public health interest (Giri et al., 2010). Therefore, understanding the composition of exosomes is important for understanding how MTB communicates with the host and for developing effective TB diagnosis and treatment strategies (Schorey and Harding, 2016).

As reported in this review, several pieces of evidence have stressed the importance of host miRNAs (Behrouzi et al., 2019), sRNAs (Coskun et al., 2021), and circular RNA (circRNAs) (Wang et al., 2023) in TB. However, more research is needed, particularly in identifying the exosomal miRNAs from MTB. Despite sRNAs having minimal impact on infection and expression rates, MTB specifically produces exosomal miRNAs crucial for modulating the host immune response. These miRNAs can alter several macrophage functions, assisting the pathogen in evading the immune response and perpetuating infection. The influence of exosomal miRNAs on TB's expression and infection process is highly significant and informative for understanding the disease. Shedding light on exosomal miRNAs is crucial as they are potential diagnostic markers and therapeutic targets in TB.

Furthermore, TB diagnosis has improved by considering associated diseases (Condrat et al., 2020). miR-29a is implicated in distinguishing the active form of TB from the latent one, thereby enhancing diagnostic accuracy (Sinigaglia et al., 2020). The potential to apply this approach during therapy is rather significant. Indeed, the possibility of establishing treatment for a critical issue of antibiotic resistance can be evaluated by examining the regulations of different miRNAs. Moreover, miR-125b is crucial for promoting MTB growth by regulating inflammatory signaling and can be safely depleted to boost the host's immunity against the infection (Sun et al., 2021a).

Studies have shown that some miRNAs may be targeted for therapy, as reported in Table 3. Unfortunately, there is scarce available data regarding their involvement in the prognosis of TB. More studies are required to elucidate their significance in diagnostic evaluation and understanding the causative agent of TB (Wang et al., 2023). According to Ma et al. (2020) increasing the concentration of miR- 155 increases the activity of the macrophage-related gene, boosting the ability of macrophages to

counteract MTB. miR-125b is widely acknowledged as one of the miRNAs that can suppress inflammation. Thus, miR-125b is widely acknowledged to suppress inflammation; thus, blocking miR-125b will prevent the suppression of the host's immune response. miR-21 plays a vital role in controlling apoptosis and immunity, so suppressing its activity may positively affect immune response capacity and inflammation degree. Modulating specific miRNAs can significantly impact TB progression, with beneficial miRNAs counteracting detrimental ones (Schröder, 2022).

Thus, there is great hope for further developing miRNA investigations related to TB. With the potential for developing miRNA studies in TB, exciting avenues may be explored in the future. Effective, high-throughput miRNA sequencing techniques are anticipated to provide greater insight into miRNA function in TB, alongside advancements in bioinformatics data analysis methods. This progress will likely lead to new technologies in diagnosis and treatment.

Conclusion

These miRNAs are critically involved in managing the immune system's response to TB, a significant global health concern. These small non-coding RNAs, including miR-155, miR-125b, and miR-29a, play a pivotal role in governing innate and adaptive immunity. They modulate the differentiation of macrophages and T cells and the levels of cytokines. Thus, exosomal miRNAs secreted by MTB are reported to have a crucial function in regulating the host's immune response, allowing the pathogen to avoid elimination by the immune system and persist in the body. Due to the exosome's stability and non-invasive nature, miRNAs have substantial value as biomarkers for distinguishing between active TB and LTBI, contributing to the development of efficient and accurate diagnostic tests. Moreover, given the diverse roles of miRNAs, miRNA-based therapies hold promise for improving immune responses and countering bacterial survival strategies. These approaches offer new ways to combat drug resistance and enhance patient treatment outcomes.

Author contributions

FM: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. AG: Data curation, Writing – original draft, Writing – review & editing. NB: Formal analysis, Investigation, Writing – review & editing. MF: Formal analysis, Investigation, Writing – review & editing. MD: Data curation, Investigation, Writing – review & editing. MA: Conceptualization, Data curation, Writing – review & editing. MZ: Conceptualization, Formal analysis, Supervision, Writing – review & editing. DN: Supervision, Validation, Writing – review & editing. RDM: Project administration, Resources, Supervision, Validation, Writing – review & editing. GPP: Conceptualization, Validation, Writing – original draft, Writing – review & editing.

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Insight into the emerging insect to human pathogen *Photorhabdus* revealing geographic differences in immune cell tropism

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Background: *Photorhabdus asymbiotica* is a species of the insect pathogenic *Photorhabdus* genus that has been isolated as an etiological agent in human infections. Since then, multiple isolates have been identified worldwide; however, actual clinical infections have so far only been identified in North America, Australia, and Nepal. Previous research on the clinical isolates had shown that the strains differed in their behaviour when infecting cultured human cells.

Methods: In this study, we investigate the differences between the pathogenic activities of *P. asymbiotica* isolates from different geographic locations. Pathogenicity was analysed using infection assays with both cultured cell lines (THP-1, CHO, and HEK cells) and primary immune cells, and peripheral blood mononuclear cells (PBMCs) isolated from human blood.

Results: Here, we present the findings from the Australian (Kingscliff) and North American (ATCC43949) clinical isolates, and non-clinical soilborne nematode isolates from Thailand (PB68) and Northern Europe (HIT and JUN) of *P. asymbiotica*. We also show the first findings from a new clinical isolate of *P. luminescens* (Texas), the first non-*asymbiotica* species to cause a human infection, confirming its ability to infect and survive inside human immune cells.

Conclusion: Here for the first time, we show how *P. asymbiotica* selectively infects certain immune cells while avoiding others and that infectivity varies depending on growth temperature. We also show that the tropism varies depending on the geographic location a strain is isolated from, with only the European HIT and JUN strains lack the ability to survive within mammalian cells in tissue culture.

KEYWORDS

***Photorhabdus*, emerging pathogen, human lymphocytes, geographical localisation, tropism**

Introduction

Members of the genus *Photorhabdus* are entomopathogenic bacteria, which form an obligate symbiosis with the insect parasitic nematode, *Heterorhabditis*. The free-living stage of the nematodes, infective juveniles, are found in the soil carrying *Photorhabdus* in their gut, where they search for insect larvae. Once found, they will burrow into the insect before

regurgitating the *Photorhabdus* which essentially acts as a "bioweapon." The bacteria release a large range of toxins and enzymes which kill the insect allowing them to replicate. The nematode then begins hermaphrodite replication cycles, using the bacterial biomass as a food source. The speed and efficiency of the nematode-*Photorhabdus* partnership in killing insect larvae, generally under 48 h (Forst et al., 1997), have made it an effective and widely used bio-pesticide.

While most species of *Photorhabdus* are only able to infect insects, being unable to grow above 34°C, one species has been reported to cause human infections, *Photorhabdus asymbiotica*. Like their insect restricted relatives, the bacteria are carried by symbiont *Heterorhabditis* nematodes. Of all reported cases of *Photorhabdus* infections, only one case did not involve a member of the *P. asymbiotica* species. In this case, an isolate of a novel strain of *P. luminescens* was found to have infected a neonate in Texas that was abandoned just hours after birth on a patch of soil. This strain is currently poorly characterised; however, it is unknown whether it would be able to infect an adult or merely took advantage of the neonate's weakened immune system. It is perhaps telling, however, that this strain, unlike the majority of other *P. luminescens* isolates, can grow at 37°C (Figure 1). Infections of *Photorhabdus*, known as Photorhabdosis, typically start with a large lesion generally on an extremity believed to be initial site of infection. If untreated secondary lesions appear on other parts of the body and in some cases, bacteria were found in the respiratory system and the heart causing endocarditis, confirming the ability of *Photorhabdus* to disseminate throughout body. It has been hypothesised they may achieve this by hijacking immune cells and essentially "hitchhiking" via the lymphatic system. This facultative intracellular infection behaviour is seen in the phylogenetically closely related *Yersinia pestis*, which are carried inside phagocytes around the body (St John et al., 2014). It is unknown how *Photorhabdus* enters the body of the human host; however, the most likely hypothesis is that an infective juvenile accidently burrows into the dermal layer of the human skin. While the *Photorhabdus* can establish an infection, the nematode would not likely be able to survive core body temperature and would need to remain near the cooler surface of the host. Some related nematodes such as *Strongyloides stercoralis* have been shown to be able to burrow through human skin (Gang et al., 2020). Nevertheless, a *Heterorhabditis* nematode has never been isolated from one of the human infections, although no one has purposely looked for them to date.

Interestingly, so far incidences of human infection have been geographically isolated, only being reported in eastern Australia, continental eastern North America, and a single case contracted by a traveller in Nepal (Farmer et al., 1989). In these cases, many of the infections were clustered in geographically constrained areas of those countries, for example, Texas in North America and Victoria in Australia. Both these areas have sandy soils which the *Heterorhabditis* nematodes seem to prefer, and infections seem to coincide with warm, wet weather, which may stimulate nematode activity or perhaps insect

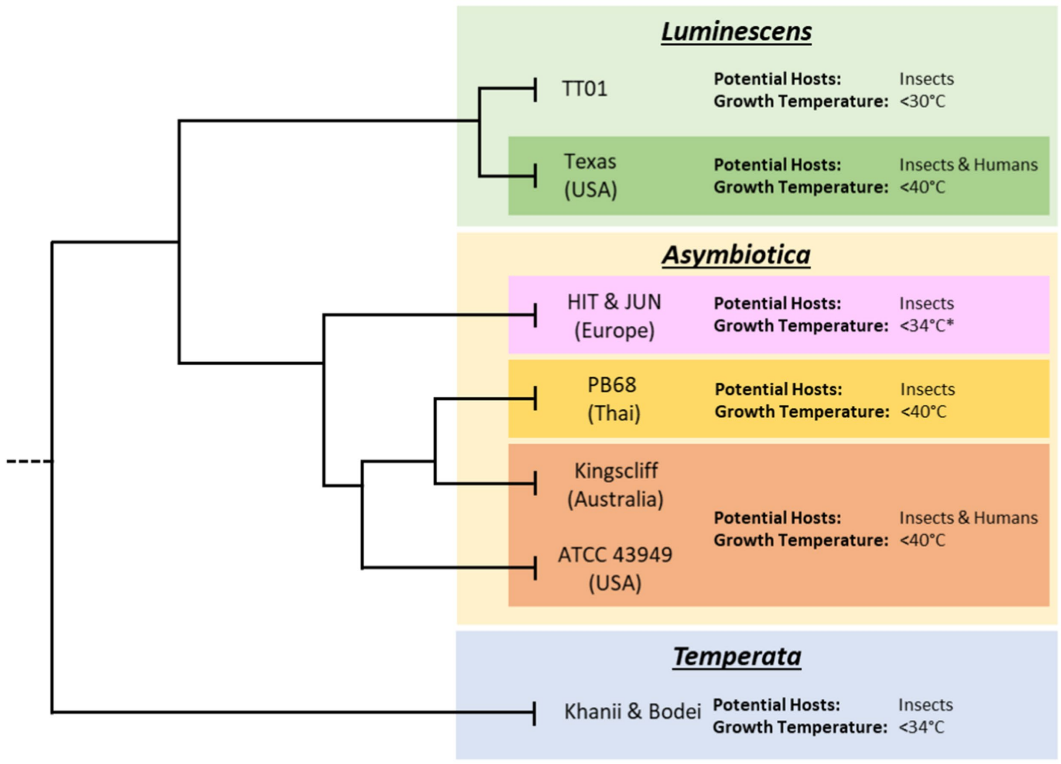

FIGURE 1
Subclades of the various *Photorhabdus* species. Tree lengths are not drawn to scale and for illustrative purposes only. Displayed are the approximate thermotolerance and known potential hosts of archetypal strains of *Photorhabdus* within each species. Data on thermotolerance are from Mulley et al. (2015) and our own observations from working with our strain collection. While published data on the HIT and JUN strains showed inability to grow at 37°C, during this project it was found that given extended growth times, of 48 h compared to 24 h, they would regularly reach higher ODs and eventually reach stationary phase.

host availability. While the number of recognised human infections is low, it should be noted that until as recently as 2017, *Photorhabdus* was not included in the standard databases used by many medical professionals to identify infections. This may have led to it being commonly mistakenly identified as a different species of bacteria. When tested in a lab setting using a VITEK 2 Gram-negative identification card, it indeed did lead to misidentification, in this case as *Pseudomonas fluorescens* (Weissfeld et al., 2005). The most accurate way to identify Photorhabdus infections is through the use of 16S ribosomal DNA gene or *recA* sequencing. However, this is time-consuming, typically taking 48–72 h (Boyles and Wasserman, 2015). A simple alternative is by visible inspection for bioluminescence by dark adapted eyes as *Photorhabdus* is the only known terrestrial bioluminescent bacterium. These factors, along with the ease that most *Photorhabdus* infections can be treated, using a standard antibiotic course, mean that many *Photorhabdus* infections are likely to have gone underdiagnosed. This may be the reason why we have only had reports of infections in the USA and Australia, as other places where *P. asymbiotica* has been found, such as rural Thailand, may not have the resources, infrastructure, or requisite knowledge to properly identify infections. In reality, infection clusters may actually only reflect the presence of clinicians who are aware of this pathogen.

Photorhabdus asymbiotica strains have all been reported to grow at human body temperature, with some strains being able to grow at up to 42°C. However, while it has been a long time since the first recorded case of a *Photorhabdus* human infection, 1977 (Farmer et al., 1989), there has been little study on this emerging human pathogenic species of *Photorhabdus.* Thus, it is unknown whether temperature tolerance is all that separates the human infective strains from the non-human infective strains. Some differences can be found in the genomes between the *P. asymbiotica* and the non-human infective *P. luminescens* strain TT01. For example, *P. asymbiotica* encodes a more limited range of insect toxicity genes, resulting in a smaller genome, approximately 600,000 base pairs [bp] less than the *P. luminescens* type strain TT01. Notably, *P. asymbiotica* lacks a homologue of the type 3 secretion system (T3SS) effector *lopT* gene possessed by all *P. luminescens* so far examined. The T3SS LopT has been shown to prevent phagocytosis (Brugirard-Ricaud et al., 2005). In the same locus as *lopT*, *P. asymbiotica* instead carries a homologue of the *exoU* gene from *Pseudomonas aeruginosa*. The ExoU toxin is a phospholipase associated with acute lung injury (Pankhaniya et al., 2004).

The only research published on the interaction between *P. asymbiotica* and tissue culture cells confirmed that it is capable of invading human immune cells and resists killing by humoral factors such as the complement system (Costa et al., 2009). This research did bring up an interesting observation, however, that the American and Australian strains of *P. asymbiotica* differed in their ability to invade human cells, despite both being human pathogenic strains. This raises the question of whether geographically distinct strains of *Photorhabdus* differ in their strategies for evading/resisting the immune system response. So far, all the research on *P. asymbiotica* have only been done using single isolates of the confirmed human infective American and Australian strains. It should also be noted that previous research into *P. asymbiotica* was performed using *Photorhabdus* cultures that had been grown at 28°C prior to cell challenge experiments. In previous studies, *Photorhabdus* has been shown to only express certain genes when grown at human body temperature of 37°C compared to the more insect-relevant body temperature of 28°C (Hapeshi et al., 2020). This presents the distinct possibility that the initial growth temperature prior to infection may influence the bacterial behaviour and its ability to establish an infection.

To get a clearer understanding of how geographically distinct strains of *Photorhabdus asymbiotica* differ in their strategies for evading/resisting the immune system and how this behaviour is affected by growth temperature, five geographically distant *P. asymbiotica* strains were studied alongside the newly identified human infective *P. luminescens* strain (Figure 2). The strains studied were the North American *P. asymbiotica* subsp. *P. asymbiotica* strain ATCC43949, the Australian *P. asymbiotica* subsp. *Australis* strain (Kingscliff), the closely related Thailand isolate *P. asymbiotica* subsp. *Australis* strain (PB68), the two Northern European *P. asymbiotica* subsp., designated HIT and JUN, and finally the recent clinical isolate of *P. luminescens* (Texas). Kingscliff, ATCC43949, and Texas are clinical isolates from confirmed human infections, while the other *P. asymbiotica* genospecies strains were isolated from soil dwelling nematodes, and thus, it is unknown whether they are capable of causing clinical infections. The lab passaged *P. luminescens* TT01-DJC strain was also included as a non-human infective negative control. To understand how the behaviour of these strains compared, they were tested for their ability to survive or avoid phagocytosis in the human monocyte derived macrophage model cell line, THP-1. We also examined how they interacted with a more natural and complete model of the human immune system. To do this, we studied the interactions of these strains with human-derived PBMCs taken from healthy human volunteers (Singapore).

Here for the first time, we reveal how different strains of *P. asymbiotica* selectively associate with certain immune cell types while avoiding others. We also show that the range of immune cells that become infected varies depending on the geographic location that the strain was isolated from. This study also confirmed the hypothesis that the Northern European genospecies strains of *P. asymbiotica*, HIT and JUN, lack the ability to survive within mammalian cells, indicating that unlike the previous assumption, not all members of *P. asymbiotica* genospecies clade are capable of infecting mammals. We also show evidence that the clinical strain of *P. luminescens* varies greatly in its behaviour compared to that of the non-human infective *P. luminescens* but still acts differently to the *P. asymbiotica*.

Results

Both the growth temperature and the specific strain of *Photorhabdus asymbiotica* greatly influence their ability to invade cultured mammalian cells

We studied the effect of growth temperature to afford a basic understanding of the behavioural host cell tropism between different *P. asymbiotica* strains. The five strains investigated (see above) were tested for their ability to invade and/or survive inside human THP-1 cells, which had previously been differentiated into a macrophage-like cell line, using gentamycin protection assays. In brief, gentamycin protection assays provide information on the ability of bacteria to invade and survive within cells. In this case, the bacteria were incubated with the eukaryotic cells at a

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Bacterial isolate	Species	Country of origin	Grow above 30°C?	Human infections?
TT01 (Lab Strain)	*P.luminescens* SubSp *laumondii*	Trinidad and Tobago	No	No
Texas	*P.luminescens*	USA (Texas; Huston, Clinical)	Yes	Yes
HIT	*P.asymbiotica* SubSp	Northern Europe (Sweden)	?	No
JUN	*P.asymbiotica* SubSp	Northern Europe (Netherlands)	?	No
PB68	*P.asymbiotica* SubSp *Australis*	Thailand	Yes	?
Kingscliff	*P.asymbiotica* SubSp *Australis*	Australia (Clinical)	Yes	Yes
ATCC 43949	*P.asymbiotica* SubSp *Asymbiotica*	USA (Clinical)	Yes	Yes

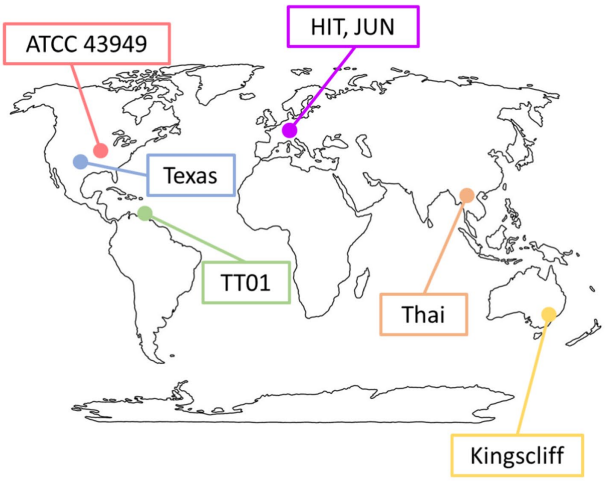

FIGURE 2
Global distribution of *Photorhabdus* strains used in this study. Table shows the *Photorhabdus* isolates used for the experiments in this study. (Clinical) indicates the isolate used was isolated directly from a human infection; otherwise, the isolate was isolated from soil dwelling nematodes in the region. A question mark indicates that not enough research has been done previously to confirm or deny the attribute.

multiplicity of infection (MOI) of 1:50 (cell:bacteria) for 2 h, after which any external bacteria still exposed to the surrounding media were killed using gentamycin (which cannot enter the eukaryotic cell). Subsequently, the gentamycin was removed by washing, and the eukaryotic cells were lysed, releasing any intracellular bacteria. These were enumerated by colony counts on agar after 24 h. Gentamycin protection assays provide an indicator of the ability of bacteria to be internalised and survive within a host cell as any bacteria remaining exposed to the extracellular milieu are killed by the antibiotic. Prior to exposure to the differentiated THP-1 cells, the bacteria were grown either at 28°C or 37°C to mid-log phase (approximate $OD_{600} = 0.4–0.6$). In addition to the THP-1 cell gentamycin protection assays, we also examined interactions with the human kidney-like cell line HEK 293 T and the hamster ovarian line CHO. As HEK 293 T and CHO cells are not professional phagocytes, these assays provided information on the bacteria's ability to actively invade cells, as opposed to simply being able to escape killing when phagocytosed.

An important observation from these experiments is that prior growth temperature of the bacteria strongly affected the ability of certain strains of *P. asymbiotica* to invade these representative mammalian cells (Figure 3).

An analysis of the cell type tropisms and "invasion" rates of the different *P. asymbiotica* strains supported previous published data on the interaction of *P. asymbiotica* with THP-1 cells. For example, the Australian Kingscliff strain had significantly higher invasion rates than the American ATCC 43949 strain, or indeed all the other *P. asymbiotica* strains tested, regardless of the prior growth temperature (Figure 3A). The increased ability to invade mammalian cells was consistent with the HEK 293 T cell assays; however, this was only the case if the bacteria were grown at 37°C prior to the challenge. Interestingly, in CHO cells, Kingscliff exhibited the same invasion rates as ATCC 43949. Having said this, both strains showed significantly lower invasion rates than the Thai PB68 strain which, in the other cells types, mirrored the behaviour of ATCC 43949. The European strains HIT and JUN demonstrated no ability to invade or survive in the host cells, which is perhaps not surprising given their inability to easily grow and replicate above 34°C. Survival times inside cells could not be determined due to unrestricted growth of bacteria and host cell death.

Interestingly, the *P. luminescens* Texas strain was also able to survive within the THP-1 cells, supporting the hypothesis that it is indeed an emerging human pathogen. Its internalisation and survival rates were similar to that of PB68, being slightly higher though not significantly so. While the data are not shown here, the lab strain of *P. luminescens* TT01-DJC was not able to survive within the phagocytes, although this is likely due to it being unable to replicate at the required THP-1 incubation temperatures.

Despite being a clinical isolate, temperature did not have a significant effect on the ability of the ATCC43949 strain to invade cells. Conversely, growing the Kingscliff strain at 37°C prior to exposure to host cells significantly increased the ability for it to invade both HEK and THP-1 cells. Interestingly, while the invasion propensity of Kingscliff previously grown at 28°C into the THP-1 cells was higher than the other *P. asymbiotica* strains, when exposed to HEK cells, the invasion rate of 28°C grown cells was similar to that of the others strains, only becoming significantly higher when grown at 37°C. Strain PB68 showed an opposite trend in comparison with that of Kingscliff, exhibiting a slight increase in invasion propensity when grown at 28°C, in comparison with when it was grown at 37°C. Nevertheless, these differences in trends did not prove significant in these experiments.

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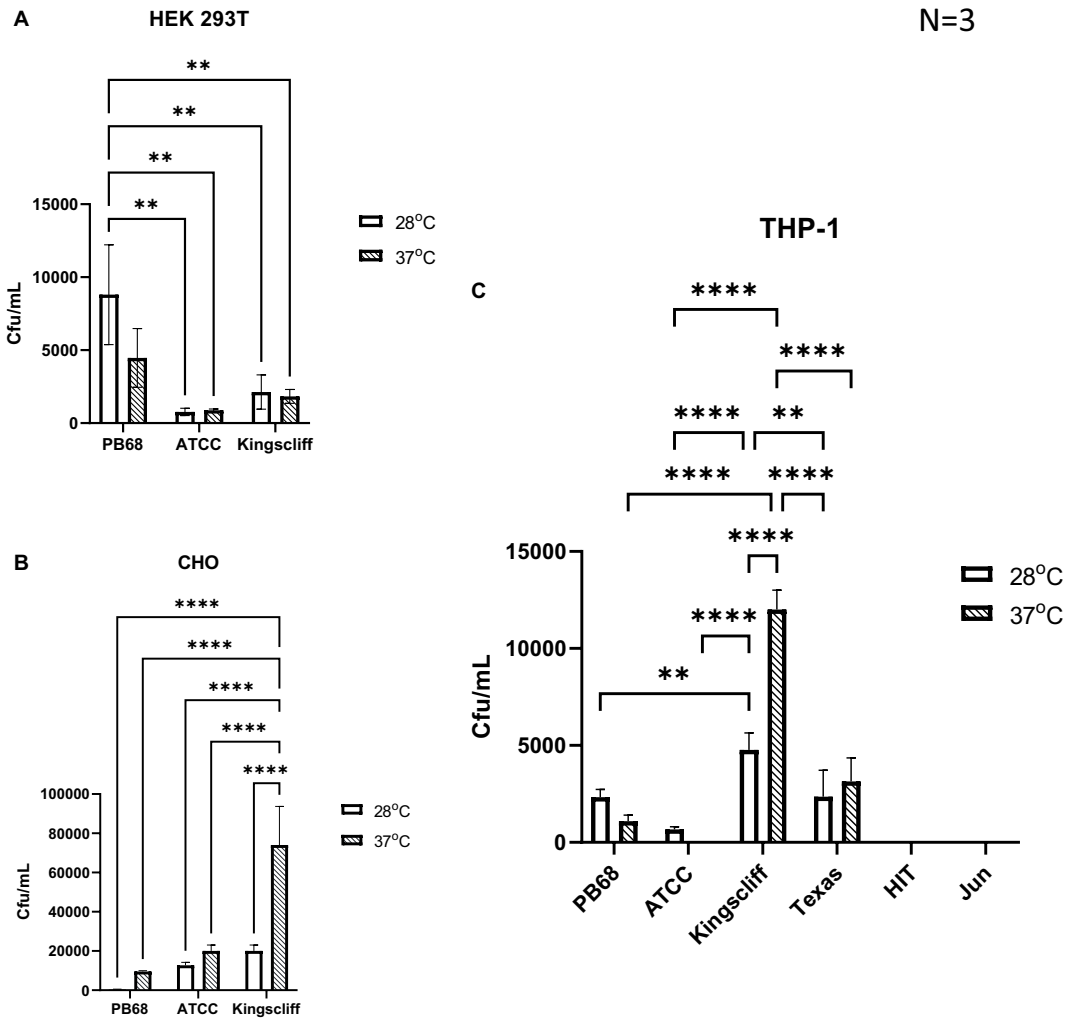

FIGURE 3
Invasion and survival of *P. asymbiotica* species inside mammalian cells. Gentamycin-based invasion assays were performed on several mammalian cell lines using the different bacterial isolates. Bacteria were grown at either 28°C or 37°C prior to assay. During the assay, the bacteria were allowed to interact with the mammalian cell lines for 2 h, after which gentamycin was added to kill any extracellular bacteria. The mammalian cells were subsequently lysed, and surviving (presumably intracellular) bacterial numbers assessed using colony-forming count plating assays. The infection assays were done with **(A)** HEK 293 T, **(B)** CHO cells, and **(C)** THP-1 cells that had been differentiated into macrophage-like cells using PMA, prior to infection. (two-way ANOVA, **<0.01, ****<0.0001, *n* = 3 biological replicates). HIT and JUN were not tested in CHO and HEK 293 T cell lines.

Temperature-dependant internalisation and/or attachment of different strains of *Photorhabdus asymbiotica* with THP-1-derived macrophages

While the invasion assay findings described above revealed specific temperature-dependant tropisms of the different *Photorhabdus* strains, they do not give a truly representative picture of what likely happens in a real infection. These experiments only tell us whether the bacteria are being internalised and surviving over a short time period in highly artificial tissue culture conditions. Therefore, we decided to use microscopy to investigate the interaction of the strains with these phagocytic THP-1-derived macrophage cells in more detail. To this end, the gentamycin protection assays were repeated as before, but instead of lysing the phagocytes after the incubation time, the samples were imaged using an inverted fluorescent microscope. For these experiments, we used GFP-labelled variants of the different *P. asymbiotica* strains. While this was possible for Kingscliff and PB68, we were not able to transform the European strains with the marker plasmid. We therefore used the non-fluorescent wild-type strains for assays with these strains and relied on phase contrast imaging to reveal their location. From the previous infection assays, it may have been expected that the European HIT and JUN strains might either simply attach to the surface of the THP-1 cells or not interact at all. The latter hypothesis was indeed the case for the JUN strain, where at either temperature the bacteria could not be seen to associate with the THP-1 cells. This may suggest that they are actively avoiding the THP-1 cells (Figure 4). Interestingly, however, the HIT strain was seen to attach to the THP-1 cells, when grown at either temperature, with possible internalisation at 37°C, although without a fluorescent label we cannot be confident about that observation (Figure 4).

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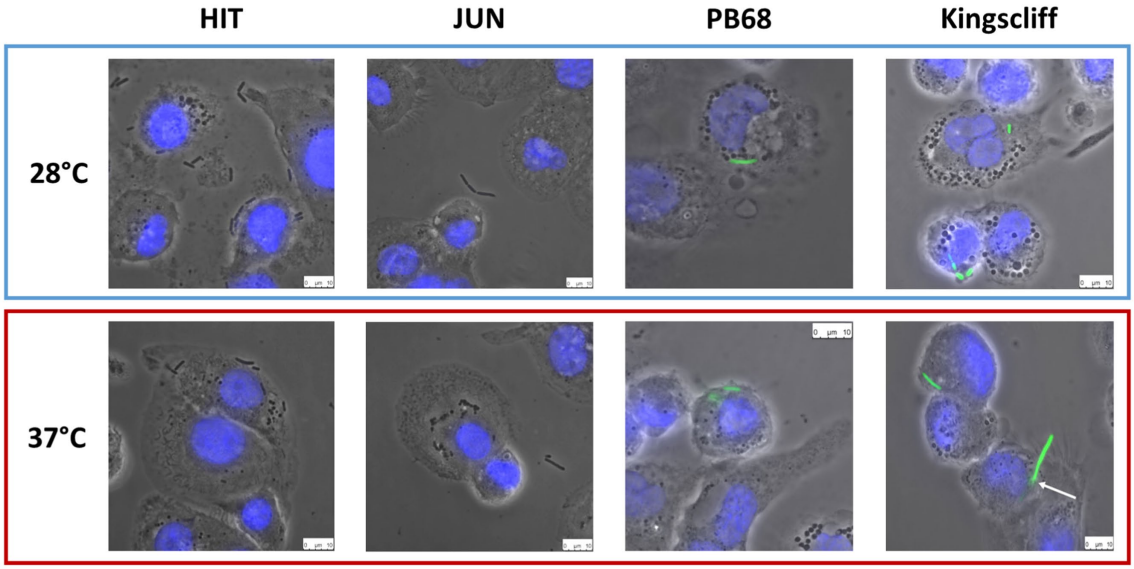

FIGURE 4
Phase contrast images of *Photorhabdus asymbiotica* subsp. strains; HIT, and JUN and fluorescent imaging of Kingscliff and PB68 with THP-1 cells. Before the experiment the THP-1 cells were seeded into 24 well plates on glass coverslips, and differentiated into macrophage like cells using PMA. Bacteria were grown O/N at either 28°C or 37°C prior to infection and allowed to infect for 2 h, at 37°C, at a MOI of 1:50. After infection the THP-1 cells were washed to remove non-internalised/attached bacteria prior to imaging. THP-1 cell nuclei were stained with DAPI (blue) while the Kingscliff and PB68 strains were constitutively expressing GFP (green).

Conversely, microscopy studies of the infection assays for both Kingscliff and PB68 clearly demonstrated that they were being internalised in the THP-1 cells when previously cultured at either temperature (Figure 4). A further interesting observation was that if Kingscliff was grown at 28°C, on occasion long bacterial filaments could be seen apparently crossing the THP-1 cell membrane, indicating that it is either invading or emerging from the cell (Figure 4). This leads us to suggest that it is using an active mechanism for invasion rather than simple phagocytosis/escape by the THP-1 cell. It was not clear from these assays if the observed bacterial filaments represented chains of single cells or single long multinucleate "hyphae."

While there are examples of bacteria that can escape the phagosome after engulfment, another strategy often seen in various pathogens demonstrates that they have adaptations which allow them to remain inside the vesicles but prevent phagolysosome maturation and so avoid their destruction. In previous unpublished work with an Austrian isolate of *P. asymbiotica*, researchers commented that transmission electron microscopy was used to confirm that in the insect professional phagocytes (haemocytes), this strain could be observed within phagosomes for at least ~2 h post-infection. However, the light microscopy methods we used in this study could not discern if the bacteria were enclosed in phagosome vesicles or free in the cytoplasm. Before the experiment, the THP-1 cells were seeded into 24-well plates on glass coverslips and differentiated into macrophage-like cells using PMA. Bacteria were grown O/N at either 28°C or 37°C prior to infection and allowed to infect for 2 h, at 37°C, at a MOI of 1:50. After infection, the THP-1 cells were washed to remove non-internalised/attached bacteria prior to imaging. THP-1 cell nuclei were stained with DAPI (blue), while the Kingscliff and PB68 strains were constitutively expressing GFP (green).

Internalisation of *Photorhabdus asymbiotica* requires actin rearrangement

As we confirmed that certain *P. asymbiotica* species are capable of entering a range of cell types, we wanted to better understand the processes involved in cell entry. Many bacteria facilitate uptake and entry into host cells by manipulating host cell actin rearrangements. In non-phagocytic host cells, this involves using either so called "zipper" or "trigger" style mechanisms (O Cróinín et al., 2012), while in phagocytic cells, host-medicated phagocytosis can be used to gain initial entry. Rearrangement of the host cell actin cytoskeleton and polymerisation of F-actin at the site of the bacterial attachment is a common feature of these processes. Cytochalasin-D is a fungal toxin that binds tightly to the end of actin filaments thus acting as a potent inhibitor of actin polymerisation, and thus phagocytosis. To determine whether actin rearrangement is important for the entry of *Photorhabdus* into host cells, THP-1 cells were treated with Cytochalasin-D prior to infection with either Kingscliff or PB68, using the methods consistent with the infection assays described above.

Inhibition of actin polymerisation by Cytochalasin-D caused a significant reduction in the abilities of both Kingscliff and PB68 bacteria to invade the cells (Figure 5). Perhaps not surprisingly, this differential effect was only seen when the bacteria were cultured at the temperature at which they had previously been shown to be most invasive at in the previous THP-1 invasion assays (Figure 4). For example, as described above, the Kingscliff strain can invade THP-1 cells but only when cultured at 37°C prior to infection. When grown at 28°C, the addition of Cytochalasin-D made no difference to any observed ability to "invade" cells. However, it should be noted that in these assays, the recovery levels of the bacteria were approaching the lower range of sensitivity limit for the CFU counts, being only

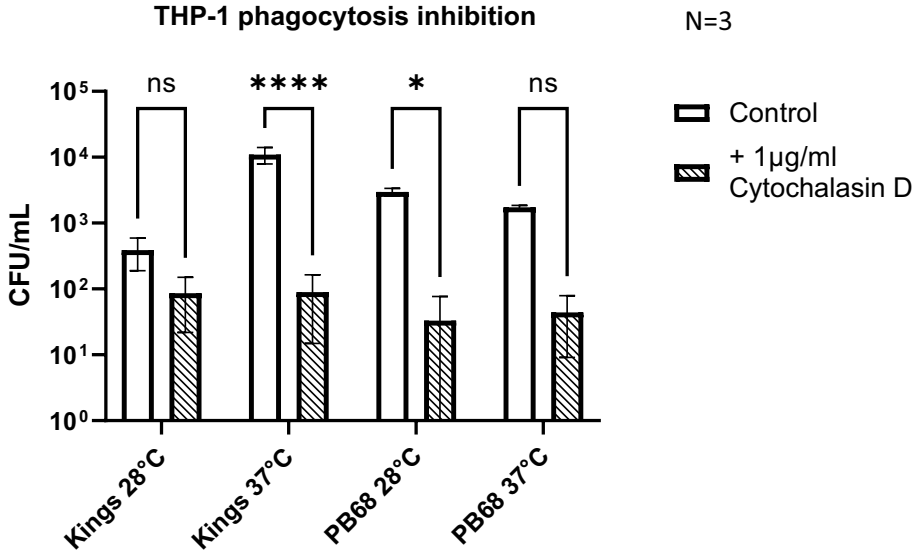

FIGURE 5
Photorhabdus asymbiotica subsp. *Australis* strains, Kingscliff, and PB68, with phagocytosis inhibited THP-1 cells. THP-1 cells were activated by PMA 48 h prior to experiment. Bacteria were grown at either 28°C or 37°C prior to infection and allowed to interact for 2 h. Prior to the infection assay, phagocytosis inhibitor cytochalasin-D was added to the THP-1 cells (*t*-test, *<0.05, ****<0.0001, $n = 3$ biological replicates).

1.6×10^1. Strain PB68, on the other hand, showed the converse of what was seen for the Kingscliff strain, with a significant reduction in invasion only at 28°C. Nevertheless, while we did still see a trend for a drop in the number of recovered bacteria in the 37°C grown samples for strain PB68, upon the addition of Cytochalasin-D, it was not significant.

Interactions of *Photorhabdus* with peripheral blood mononuclear cells (PBMCs)

The assays described above investigating *P. asymbiotica* interactions with mammalian cells were performed using immortalised cultured cells. Even in the case of cell lines such as the differentiated THP-1 macrophage-like cells, this is not a perfect model of how the bacteria may behave when exposed to the more complicated and diverse human cellular immune system. A large part of the immune system response comes from the PBMCs which are round nucleated white blood cells found circulating in the blood. The PBMCs consist of lymphocytes (T cells, B cells and NK cells), monocytes, and dendritic cells. These cell types represent both the early innate immune response, phagocytosis, antigen presentation activities by dendritic cells and tissue sequestered monocytes, and the late adaptive immune response, through the activation of T and B cells (and subsequent antibody production and immune memory). Thus, PBMCs taken from healthy human volunteers represent an excellent resource for studying the interactions of *P. asymbiotica* with a "natural" more complete cellular immune component model.

In order to determine which PBMC cell types, *P. asymbiotica* interacts with, either through adhesion or internalisation, constitutive GFP expressing strains of the; Australian (Kingscliff) and Thai (PB68) *P. asymbiotica* were used alongside a non-clinical *P. luminescens* strain (TT01-DJC), which provided a suitable negative control. Attempts were made to create a GFP expressing strain of the American *P. asymbiotica* (ATCC 43949) as well, but as with the HIT and JUN strains, it proved recalcitrant to transformation. The GFP expression strains were grown at either 28°C or 37°C and then allowed to interact with freshly harvested human PBMCs for 2 h, before analysis using flow cytometry (Figure 6A). To investigate whether any of the observed interactions were either bacterial or PBMC activity-dependant, control samples of these bacteria were killed prior to addition to the PBMCs. These controls allowed us to distinguish between passive phagocytic ingestion as opposed to active virulence processes. The flow cytometry allowed not only for the identification of the different PBMC cell types, through detection of differentiating surface markers (Figure 6B), but also which were infected with *Photorhabdus* by identifying the GFP signal associated with each cell (Figure 6C).

The findings from these experiments build upon our observations from the tissue culture invasion assays, indicating that the different *Photorhabdus* strains exhibit unique responses to the different PBMC cell types. Surprisingly, *P. asymbiotica* PB68 and *P. luminescens* TT01 showed very similar cell type interaction profiles (Figures 7B,D). Both had low levels of association with the majority of the PBMCs cell types, ~0–30%, which dropped further for PB68 when grown at 37°C. There was also no significant change in the cell association profiles when the bacteria had been killed prior to exposure to the PBMCs, suggesting phagocytosis or passive cell surface binding is in operation. The only cell type that showed high levels of bacterial association were the dendritic cells, ~70–90% at 28°C. Importantly, there was a significant drop for the pre-killed PB68 samples, though the number of GFP associated cells was still significantly higher than the other PBMCs cell types at this temperature, the drop was much larger for the TT01-DJC strain, dropping down to that of the other PBMC cell types.

Unlike the other two strains tested, the Australian *P. asymbiotica* clinical strain (Kingscliff) showed very different behaviour in its

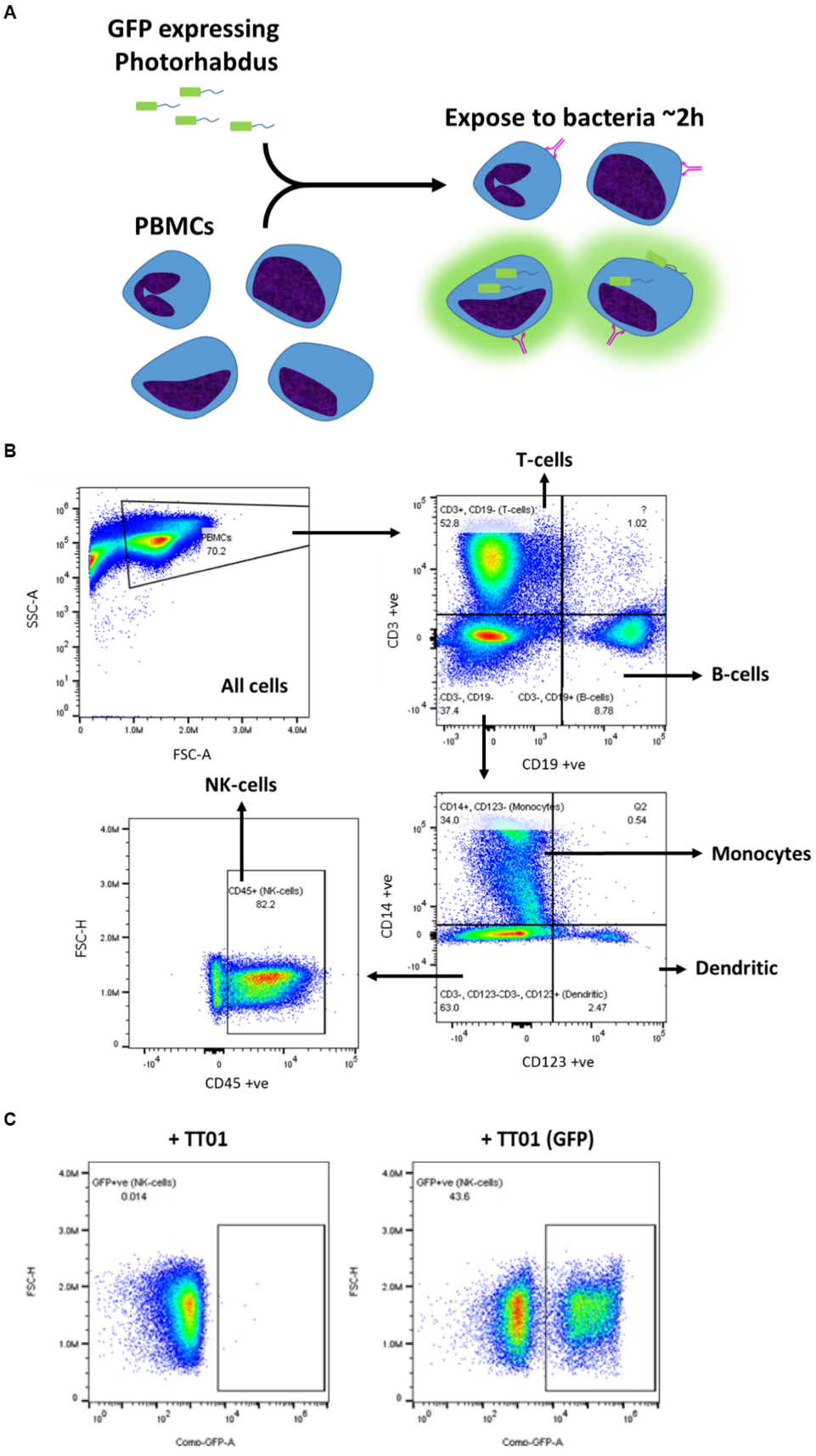

FIGURE 6
Flow cytometry strategy for identification of *Photorhabdus*-infected PBMCs. **(A)** Human-derived PBMCs were exposed to various strains of *Photorhabdus* expressing GFP for 2 h, before analysis by flow cytometry to calculate the percentage of PBMCs infected. In theory, a GFP signal should be detected from PBMCs which have attached or internalised bacteria. The *Photorhabdus* strains were cultured first at either 28°C (simulated ambient

(Continued)

FIGURE 6 (Continued)
insect body temperature) or 37°C (human core body temperature) prior to the infection. As controls, *Photorhabdus* cultures of each strain were killed 1 h before exposure to the PBMCs. **(B)** Gating strategy for identifying the different PBMC cell types using flow cytometry. Antibody and fluorophore panel can be found in the methods section. **(C)** Alongside detecting signals for each of the antibodies, once the PBMC types were gated out, GFP signal from any internal or attached bacteria was also detected. A distinct population of the cells (in this case monocytes) can be seen to have a GFP signal (+TT01_GFP), which is not seen when PBMCs are infected with non-GFP expressing bacteria (TT01). Using this, a percentage of each PBMC cell type that exhibited a GFP signal, indicating infection with bacteria, could be calculated.

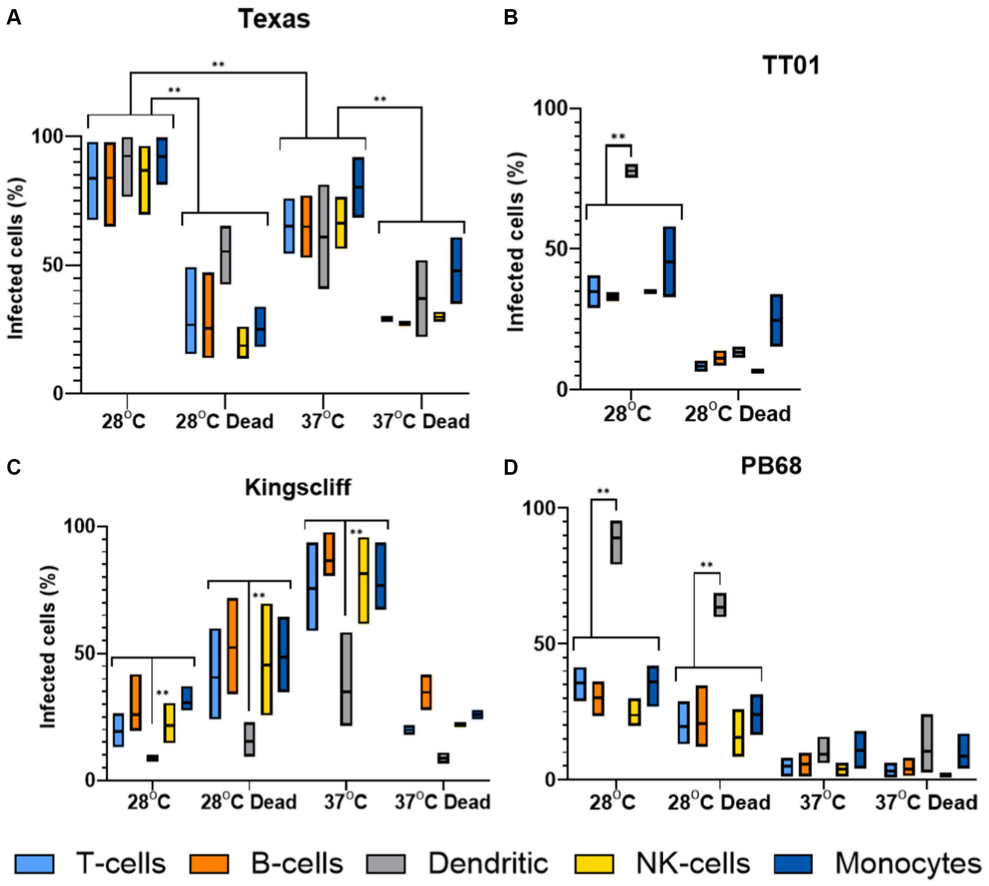

FIGURE 7
Flow cytometry analysis of varying infection rates of different strains of *Photorhabdus*. Human PBMCs were infected with different strains of GFP +ve *Photorhabdus* and analysed by flow cytometry as described in figure (three experimental replicates for each sample). Four strains of *Photorhabdus* were tested: **(A)** Texas the clinical *P. luminescens* isolate from a human neonate infection in the USA. **(B)** TT01-DJC the *P. luminescens* lab strain originally isolated from a soil nematode. For this strain data could not be obtained for 38°C due to TT01_DJC's inability to grow at this higher temperature. **(C)** Kingscliff—a *P. asymbiotica* subsp. *Australis* isolate from a human infection in Australia, and **(D)** PB68—a *P. asymbiotica* subsp. *Australis* nematode isolate from Thailand ($n = 2–4$, two-way ANOVA, **<0.01). Only relevant p-values have been shown due to the number of statistical comparisons.

associations with the various PBMC cell types. Bacteria grown at 28°C generally showed low levels of cell association, ranging from 30 to 10% for most PBMC cell types. However, this increased significantly for Kingscliff when pre-cultured at 37°C, reaching levels of 60–100%. When the 37°C cultured Kingscliff strain was killed prior to exposure to the PBMCs, association rates dropped to a similar level seen for the live 28°C grown bacteria, ~40–10%. Strangely, the pre-killed bacteria grown at 28°C actually showed a slight increase over the live cells. Unlike the other *Photorhabdus* strains, at both cultured temperatures, even if the bacteria were killed prior to addition to the PBMCs, the dendritic cell association level was significantly lower than the other PBMCs. This contrasts with the other *Photorhabdus* strains where the dendritic cells always had association levels either the same or higher than the other PBMC cell types.

Finally, the Texas strain again showed a different phenotype to all the other strains, both *P. asymbiotica* and *P. luminescens*. In this case, all PBMC cell types showed high levels of bacterial association, with no significant distinction with dendritic cells unlike the other *Photorhabdus* strains. A higher proportion of the PBMCs had bacterial association when the Texas strain was grown at 28°C instead of 37°C, with all cell types almost reaching 100%. There was also a significant decrease when the bacterial were killed prior to incubation at both temperatures.

Discussion

Here, we have presented data obtained from both cultured and human-derived *ex vivo* PBMC cells that details how prior bacterial growth temperature and host cell type strongly influence the behaviour of various strains of the *P. asymbiotica*. One of the first findings from these experiments is that the European HIT and JUN strains, which were confirmed by whole genome sequencing to be closely related genospecies to clinical isolates of *P. asymbiotica*, displayed no ability to survive within mammalian cells. Considering that they are relatively temperature intolerant, growth at 37°C was unreliable and slow, and furthermore they have yet to be associated with a human infection, this perhaps should not have been unexpected. These observations did indicate that they are not at all well-adapted mammalian pathogens, unlike the known clinical isolates. These strains are likely either avoiding phagocytosis completely or are simply being killed by the THP-1 cells. Microscopic analysis of the interaction of these two strains with THP-1 cells suggested the former explanation for JUN and the latter for HIT which was seen to be internalised but not recovered during survival assays (Figures 4, 5). As JUN is not being internalised, it suggests that it can inhibit the process such as through secreted effectors from the Type 3 Secretion System (T3SS). The T3SS deployment is a common mechanism that many Gram-negative pathogens use to manipulate lymphocytes and prevent phagocytic destruction (Santos and Finlay, 2015). However, as JUN was not seen attaching to the THP-1 cells, this would not be possible for that strain. This suggests an alternative method JUN uses to evade internalisation. We speculate that HIT remains an exclusive insect pathogen, while JUN may have some limited potential as a mammalian pathogen. Nevertheless, these findings demonstrate that even bacteria isolated from similar geographic locations, for *P. asymbiotica* genospecies isolates, can show clear differences in their behaviour.

Previous research into the clinical isolates of *P. asymbiotica* showed that an American isolate was only weakly phagocytosed and could not invade HeLa cells, unlike a representative Australian clinical isolate (Costa et al., 2009). Our data confirm these previous experiments, showing that our chosen American strain, ATCC 43949, shows only weak internalisation into either phagocytic or non-phagocytic cells, while the Australian Kingscliff strain showed high levels of internalisation (Figures 4, 7). The observation of internalisation in non-phagocytic cells for the Kingscliff strain suggests that cell invasion is an active virulence process. Conversely, ATCC 43949's low levels of internalisation in phagocytic cells suggest that it is instead actively evading of phagocytosis.

However, we expanded on these previous findings by also showing that these two strains differ in their response to growth temperature, namely, that ATCC 43949 behaviour is not affected by temperature, in contrast to Kingscliff which shows higher rates of internalisation when grown at human body temperature compared to the lower temperature more appropriate of an insect infection. These differences arise despite all incubations of the bacteria with the mammalian cells necessarily being performed at 37°C. This confirms that prior growth at different temperatures leads to phenotypic adaptations that influence the outcome of the subsequent interactions for the Kingscliff strain. It is currently unknown exactly what changes in protein expression are responsible for these adaptations, but the most likely candidates would either be exotoxins, cell surface adhesins, or capsular polysaccharides.

Interestingly, the genomes of the *P. asymbiotica* strains sequenced encode several homologues of *Yersinia* adhesion proteins, which could prove to be good subjects for further study. The selective advantage for temperature-dependant cell tropisms for Kingscliff is likely related the need to conserve resources as some of these pathogenicity factors are likely irrelevant for insect infections. Similar temperature-dependant tropisms can be seen in many insect–human pathogens (such as *Yersinia pestis*), which in fact makes the American strain the more curious of the two strains (Konkel and Tilly, 2000). It should also be noted that in the microscopic analysis of Kingscliff interactions, it is on occasion seen traversing a THP-1 cell membrane, suggesting the ability to enter or leave phagocytes though methods other than phagocytosis or cell lysis (Figure 5). Taken together, these data suggest that the Kingscliff strain may have distinct regulons for both insect and mammalian infections.

While we were unfortunately not able to test how the American strain ATCC43949 interacts with human PBMCs, we were able to determine sound findings for the Kingscliff strain (Figure 7). Once again there were large increases in bacterial association for nearly all PBMC types at the higher temperature, mirroring the behaviour seen in the cell line assays. However, the outliers were the dendritic cells, which at both temperatures showed significantly reduced bacterial association compared to the other PBMCs at either temperature. This is in contrast to the observations from the *P. luminescens* TT01-DJC strain and *P. asymbiotica* strain PB68, where dendritic cell bacterial association was generally higher than with other PBMC cell types. High levels of dendritic cell association would be expected as they are proficient professional phagocytes, therefore suggesting that Kingscliff may be actively evading the dendritic cells. Dendritic cells are also one of the key mediators of the early immune response, being responsible for presenting antigens, typically at the lymph-nodes, to activate T- and B-cell responses. Thus, by avoiding dendritic cells, Kingscliff may be delaying a rapid and lasting immune response, until it can establish a more robust infection. Surprisingly, this difference was also observed with the pre-killed Kingscliff, indicating that the method of evasion is not an active one, and may be mediated by factors such as a bacterial capsule.

The fact that we have shown that there are large differences between the two human infective strains despite both showing similar symptoms such as distal secondary lesions suggests that they have evolved different strategies to overcome the mammalian immune systems. This may not be surprising as the bacteria are geographically constrained by the need to inhabit either their soil nematode isolates or insect corpses, making migration from Australia to mainland North America unlikely.

One of the main symptoms of Photorhabdosis are secondary lesions formed distant from the primary site of infection, which is generally suggestive of a pathogen that is capable of dissemination through either the blood or lymphatic system, often by invading immune cells. A good example of this infection strategy is the plague bacterium *Yersinia pestis*, which can disseminate around the body by invading lymphatic system phagocytes. Our observations here suggest the Kingscliff strain may use a similar strategy, but as Kingscliff was seen to actively avoid dendritic cells it suggests it may preferentially invade non-phagocytic cell types. On the other hand, ATCC 43949

appeared to avoid internalisation, suggesting it may be using a strategy more similar to that of *Streptococcus pyogenes*, which instead attaches to the outside of immune cells as they travel to lymphatic nodes (Siggins et al., 2020).

One hypothesis as to why we see these differences in the American and Australian strains is that they may have different warm-blooded hosts. Humans are likely a dead-end host for *Photorhabdus*, the nematode vector being unable survive above 34°C. However, it is possible that the nematode might be able to set up a successful infection alongside the *Photorhabdus* in a small warm-blooded animal such as a mouse if it is killed rapidly. If this is the case, then varying warm-blooded hosts in each region may have led to the divergent evolution we see. Another possibility is even that while the American strains infect mammals, the Australian strains instead infect ground dwelling birds. With this in mind, it should be noted that only Kingscliff is able to readily grow up to a temperature of ~42°C, which coincidentally is the average body temperature for most avian species.

Interestingly, the recently identified first human pathogenic strain of *P. luminescens* (Texas strain) displayed different behaviour to both the American and Australian strains. Much like the ATCC 43949, growth temperature seemed to have no effect on invasion rates; this could be seen for both the THP-1 cells and PBMCs. However, in the THP-1 cells, it exhibited a slightly higher rate of invasion than the American strain, although not to the same extent as the Australian strain. However, in the PBMC experiments, the Texas strain showed a unique phenotype distinct from that of other strains where it was seen associating equally with all PBMC cell types at high levels.

This suggests that unlike the strategy used by the Kingscliff strain, which is to avoid dendritic cells, the Texas strain attacks all cell types, possibly attempting to overwhelm the immune system. This is reminiscent of how *Photorhabdus* acts in an insect infection, where the bacteria rapidly kill immune cells after establishing an infection, suggesting the Texas strain has adapted the more straight forward and aggressive approach used against the insect immune system to mammalian immune systems as well, while Kingscliff and possibly ATCC43949 have developed a distinct strategy for mammalian infection, which likely involves preventing early identification.

The final strain investigated was the Thai strain PB68, which until this point had neither been studied nor shown to directly cause a human infection. In addition to the ability of the Thai strain to grow at 37°C, it also carries a homologue of the pPAU1 plasmid of ATCC43949, only found in the clinical isolates, which suggests it is indeed capable of mammalian infection. Backing up this hypothesis, unlike HIT and JUN, PB68 was able to survive challenge by the phagocytic THP-1 cells and in fact showed higher rates of internalisation than the American strain, although not to the same level as Kingscliff. However, this could be brought into question by the observations of its interactions with the PBMCs, in which PB68 showed almost identical results to the non-human infective *P. luminescens* strain TT01-DJC. Both these bacteria had low rates of association in all cell types, apart from dendritic cells. Thus, unlike Kingscliff, these strains would likely get detected by the immune system early in the infection hampering their ability to successfully establish an infection in a mammalian host. Curiously, a major difference between the Kingscliff and PB68 strains that further hints that it may not be a well-adapted mammalian pathogen relates to the observations that it reacted in the opposite way to Kingscliff in regard to activity upon previous growth temperature. While not causing a statistically significant increased growth rate at 28°C, these conditions did lead to higher rates of invasion and association with both the cultured cells and the PBMCs. This, as is the case for the Kingscliff strain, showed that the bacteria are capable of responding to different host body temperatures, although it is not known whether this represents a strategy to "hide" from the immune system as we hypothesise for ATCC43949 or a downregulation of pathogenicity genes which normally used for insect infections. A second difference that was noted in the behaviour of Kingscliff and PB68 was opposite preferences for invasion of cultured cell types. Where Kingscliff was by far the most successful in invading THP-1 and CHO cell lines, PB68 outperformed it by a large margin in the HEK cell line. Exactly what could be the cause of these differences or the evolutionary implications is unknown. However, a likely candidate may be differing cell surface markers on these cell lines, which the bacteria are recognising.

Materials and methodology

All materials and methods used during the studies found in this thesis have been collected into this section as to make for easier reading. Where appropriate in other chapters, brief descriptions of methodology have been repeated to provide clarity and context.

Bacterial protocols

Bacterial cultures

Routine growth of bacterial strains (Table 1) was carried out in standard lysogeny broth (LB), with shaking at ~180 rpm unless otherwise stated. *E. coli* cultures were grown at 37°C, while *Photorhabdus* were grown at either 28°C or 37°C, as required for the experiment. It should be noted that *P. luminescens* were normally grown 28°C, due to having growth arrest above 34°C. However, for some experiments, a sub-culture of *P. luminescens* would be incubated at 37°C, and while the bacteria remained viable, no further growth would occur. For growth on solid media, LB was supplemented with 1.5% agar, 0.1% sodium pyruvate, and any relevant antibiotics; plates were incubated in the dark. *Photorhabdus* strains were confirmed to be sensitive to gentamycin prior to performing assays.

Antibiotics used

Various antibiotics were used during these studies for selection of transformed strains, the concentrations of which can be found in Table 2. It was observed that the *P. asymbiotica* strains had a natural resistance to ampicillin, so this was avoided to use a selective agent in plasmids where possible.

Transformations

All transformations of *E. coli* were done using the DH5a laboratory strain which was made chemically component to allow for introduction of plasmids through heat shock. Most of the transformations into *E. coli* were simply for the creation and replication of plasmids, due to its efficiency of plasmid uptake. *Photorhabdus*, on the other hand, does not seem to readily produce chemically competent cells. Thus, they were transformed using electroporation.

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TABLE 1 Bacterial and cell lines used in this study.

Strain	Source	Features
Bacteria		
DH5a (*Escherichia coli*)		
DH5a_GFP	This study	pBam7 (Constitutive GFP expression)
TT01 (DJC) (*P. luminescens* subsp. *laumondii*)	David Clarke Lab (Isolated from soil nematode in Trinidad and Tobago)	Lab strain of *Photorhabdus*. Growth range: <30°C
TT01_GFP (*P. luminescens* subsp. *laumondii*)	This study	Lab strain of Photorhabdus. Growth range: <30°C Constitutive GFP expression (pBam7)
JUN (*P. asymbiotica* subsp.)	(Isolated from soil nematode in the Netherlands)	Growth range: >30°C but limited
HIT (*P. asymbiotica* subsp.)	(Isolated from soil nematode in Sweden)	Growth range: >30°C but limited
PB68 (*P. asymbiotica* subsp. *Australis*)	(Isolated from soil nematode in Thailand)	Growth range: >30°C
PB68_GFP (*P. asymbiotica* subsp. *Australis*)	Waterfield lab	Growth range: >30°C pBam7 (Constitutive GFP expression)
Kingscliff (*P. asymbiotica* subsp. *Australis*)	(Clinical isolate from Human infection in Kingscliff, Australia)	Human infective. Growth range: >30°C
Kingscliff_GFP (*P. asymbiotica* subsp. *Australis*)	Waterfield lab	Human infective. Growth range: >30°C Constitutive GFP expression
ATCC 43949 (*P. asymbiotica* subsp. *Asymbiotica*)	(Clinical isolate from Human infection in USA)	Human infective. Growth range: >30°C
Eukaryotic cell lines		
HEK 293 T (*Homo Sapiens*)	ATCC	Human cell line isolated from embryonic kidney
CHO (*Cricetulus griseus*)	ATCC	Chinese hamster cell line isolated from ovaries
THP-1 (*Homo Sapiens*)	ATCC	Human monocyte cell line. Can be differentiated into macrophages with addition of PMA

Electroporation of *Photorhabdus*

It has been found that unlike some cells, *Photorhabdus* loses competency when frozen, thus electrically competent cells must be created on the same day as the transformation. On the day of the transformation, 100 mL of LB would be sub-cultured with 4 mL of *Photorhabdus* grown overnight. This would be left to grow, as per normal *Photorhabdus* growth conditions, till ~OD 0.2 (roughly 4 h) then be placed on ice for 90 min. The bacteria would then be pelleted at 4,000×*g*, 10 min, and 4°C and then resuspended in 100 mL of ice-cold SH buffer (5% [wt/vol] sucrose, 100 mM HEPES). The bacteria were pelleted and resuspended in increasing smaller volumes of SH buffer; 50 mL, 1.6 mL, before being finally resuspended in 160 µL of SH buffer. To pre-chilled 2 mm electroporation cuvettes, 40 µL of the cells were added. While still on ice 4 µL of DNA was added to the cells, and electroporation was carried out using the following parameters: 2.5 kV, 25 µF, and 200 Ω. After electroporation, 1 mL of LB was quickly added to the bacteria and incubated in normal *Photorhabdus* growth conditions for 1 h, after which they were plated onto LB plates with appropriate antibiotics.

TABLE 2 Common antibiotics and media supplements used during this study.

Use	Supplement	Working concentration
Antibiotics	Gentamycin	10 µg/mL^{-1}
	Ampicillin	100 µg/mL^{-1}
	Chloramphenicol	25 µg/mL^{-1}
	Kanamycin	25 µg/mL^{-1}
	PenStrep	50 U/mL^{-1}
Promotor control	Arabinose	0.2% (w/v)
	Glucose	0.2% (w/v)

Eukaryotic cell protocols

Maintenance of cultured mammalian and insect cells

Maintenance of mammalian cells was done at 37°C with a humidified atmosphere of 10% CO_2 with no shaking. HEK 293 T and CHO cells were grown in DMEM media supplemented with 10% FBS, L-glutamine, and non-essential amino acids, while THP-1 cells were grown in RPMI supplemented with 10% FBS, L-glutamine, and non-essential amino acids. Cells were generally split at approximately 70–80% confluence.

Infection assays

Mammalian cells were seeded at 2×10^5 onto sterilised glass coverslips in 24-well plates, in 500 µL of their respective cell media without antibiotics and left to adhere overnight at 37°C with a humidified atmosphere of 10% CO_2. In the case of THP-1 cells, 100 nM of PMA was added after seeding 48 h prior to the addition of bacteria to induce activation and adherence. Once the cells had fully adhered, bacteria grown to mid-log phase (OD 0.4–0.6) and resuspended in the cell media were added to each well at a MOI of 1:50 (~1×10^7). Bacterial infection of cells was conducted over 2 h, at 37°C with a humidified atmosphere of 10% CO_2. Afterwards, 200 µg/mL of gentamicin was added to each well for 1 h to kill any non-internalised bacteria. After incubation with the gentamycin, the media were subsequently removed, and the cells were washed 3× with PBS, finally being resuspended in 100 µL of 1% Triton-X-100 (in PBS) for 10 min at room temperature, to lyse the cells and release any internalised bacteria. 900 µL of LB was then added, and the cells were

homogenised by pipetting. CFU counts were then done for each well as described above in the CFU/OD assay.

Inhibition of phagocytosis

THP-1 cells were seeded and activated as specified above for an infection assay, but 2 h prior to addition of bacteria, 1 μg/mL of Cytochalasin-D was added to the THP-1 cells. Afterwards, the infection assay was carried out as normal.

Imaging of infected eukaryotic cells

Cells were grown and infected as with the infection assays above, but after the gentamycin incubation and washing steps, the cells were not lysed. Instead, the coverslips with the attached cells were transferred to new wells and fixed with 2% paraformaldehyde (PFA) for 30 min. After fixation, the cells were washed 3× with PBS and then had DNA stained using 300 nM of DAPI for 5 min. After staining, cells were again washed 3× with PBS, and the coverslips were placed down onto a glass slide and sealed with clear nail varnish. Cells were then imaged using a Leica DMi8 fluorescence Microscope.

PBMC extraction and purification

Fresh whole human blood from healthy volunteers was diluted at a 1:1 ratio with PBS-EDTA and layered onto 12.5 mL of Ficoll medium in a 50 mL tube, making sure the blood and Ficoll layer to not mix. The tube containing the Ficoll/blood layers was spun at 400×*g*, room temperature for 30 min, with acceleration and deceleration set at the minimum. This allowed the formation of a layer of PBMCs between the plasma and Ficoll, which was carefully removed being sure to not take any of the other layers. The PBMCs were washed in PBS-EDTA and spun again at 300×*g*, room temperature, for 5 min. The supernatant was removed, and the pellet containing the PBMCs was resuspended in 20 mL PBS-EDTA. The cells were then counted using a haemocytometer. PBMCs were then either used straight away or resuspended in RPMI at a dilution of approximately 20 million cells per mL for freezing. PBMCs for freezing after being resuspended had 2× freezing media added at 1:1 ratio and were aliquoted into cryotubes. The tubes were then either stored at −80°C in Mr. Frosty freezing containers or in liquid nitrogen for long-term storage.

- 2X Freezing media.
- 20% DMSO in Foetal Bovine serum (FBS).

Flow cytometry of infected PBMCs

PBMCs were defrosted at 37°C and resuspended in RPMI supplemented with 10% FBS and no antibiotics. Approximately 1×10^6 PBMCs were seeded in wells for each condition/bacterial strain, including wells for the controls of no bacteria and unstained PBMCs. Bacteria, grown to mid-log phase at either 28°C or 37°C, washed with PBS and resuspended in RPMI were added to the PBMCs at a MOI of 1:50 (cells:bacteria). For the killed bacterial assays, aliquots of the bacterial cultures were taken and killed with a mixture of 10 μL/mL PenStrep and 2 μL/mL chloramphenicol for 1 h prior to washing and addition to PBMCs. The bacteria and PBMCs were incubated together for 2 h at 37°C, after which 200 μg/mL of gentamycin was added for a further 1-h incubation. After the gentamycin incubation, the PBMCs were washed 3× in RPMI, then resuspended in 200 μL of ice-cold BSB, and transferred to a 96-well V-bottom plate.

PBMCs were spun in the plate at 400×*g* for 5 min, then resuspended in 50 μL of near-IR L/D stain (in PBS), and incubated in the dark on ice for 10 min. PBMCs were washed in staining buffer, then resuspended in 25 μL of antibody cocktail (Table 3), and incubated in the dark on ice for 30 min. PBMCs were then washed in 125 μL BSB, then 150 μL PBS, and finally resuspended in 200 μL staining buffer.

Stained PBMC samples were analysed by flow cytometry using a Cytek Aurora, with compensations having been previously generated for each antibody-fluorescence channel. The GFP signal from the bacteria was detected using the FITC channel on the instrument, with compensations for this channel being generated using PBMCs stained with a GFP conjugated antibody. This antibody was not included in the final experimental panel.

Molecular techniques

All DNA, plasmids, and primers were stored at −20°C and kept on ice when in use.

Purification of plasmids

Plasmids (Table 4) were purified from bacterial strains using the Qiagen Miniprep Spin Kit as per the manufacturer's instructions. 5 mL overnight cultures were used, and the final elution was conducted in 2 × 20 μL washes using molecular grade water.

Taq and colony PCR

When sequence reliability was not a concern such as during colony PCR or for very small amplicons, Taq polymerase was used. PCR parameters and relative volumes of reagents were done as per the manufacturers' recommendations. For rapid screening of possible

TABLE 3 Antibodies used for identification of PBMCs in flow cytometry.

Cell marker	Florescent dye
CD45	Pacific Orange
CD3	AF-700
HLA-DR	BV-786
CD56	PE-Cy7
CD19	APC
CD14	BV711
CD16	BV605
CD11c	BV421
CD123	PE
L/D	Near-IR

Cell marker represents the cell surface protein target of the specific antibody, and the florescent dye is the conjugated fluorophore.

TABLE 4 Plasmids used in this study.

Plasmid	Source	Features	Parent plasmid
pBam7		Constitutively expressed GFP	N/A

TABLE 5 Primers used in this study.

Primer	Sequence
Effector constructs	
GFP_Fw	ACGGCGGCCGCATAACTTCGTATAGCATACATTATACGAAGTTATTTATTTGTAGAGCTCATCCATGCC
GFP_Rv	GAATTCATAACTTCGTATAATGTATGCTATACGAAGTTATTAAGAAGGAGATATACATATGGCTAGCAAAGGAGAAG

transformants, a small amount of individual bacterial colonies were taken from plates and boiled in 50 µL water at 95°C for 5 min. This was then used as the template for the colony PCR.

Agarose gel electrophoresis

Quantification and identification of DNA fragment sizes were done using Agarose gel electrophoresis. 1% gels (w/v) were added to 1× Tris-Acetate-EDTA (TAE) buffer, and the mixture was microwaved until the agarose had melted. The solution was allowed to cool slightly; then, SYBER-safe gel stain was added at a dilution of 1:10,000. The agarose was poured into a mould and a left to set for approximately 20 min after which the gel was loaded with the sample, alongside the GeneRuler 1 kb Plus Ladder from Thermo Fisher, and the gel was run at 110 V for approximately 40–45 min. Visualisation of the gel was done using the Bio-Rad ChemiDoc MP Imaging System.

Primers

All primers (Table 5) used in these experiments were ordered from IDT.

Statistical tests

All statistical tests along with *p*-values used are found in the figure legends of the appropriate figure. Unless otherwise stated, all tests were done in prism using standard settings.

Conclusion

In conclusion, we have shown here that geographically distinct strains of *P. asymbiotica* not only differ in their ability to establish human infections but also in their behaviour towards temperature and different host cell types. We also show that the novel human infective *P. luminescens* Texas strain can infect and survive inside human phagocytes. It also seems to aggressively colonise human PBMCs regardless of cell type or growth temperature, unlike the human infective *P. asymbiotica*. Considering this, it brings into question what molecular adaptations allow for a *Photorhabdus* species to become human infective and how these adaptations arise and spread in a population and between species. The newly isolated Texas strain gives an excellent opportunity to study this, seemingly having only recently gained these adaptations. This knowledge will only become more important over time as bio-pesticides, such as *Photorhabdus* and its symbiont nematode, see increasing popularity.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Singapore A-Star Institute, Fusionopolis Way, #20-10, Connexis North Tower, Singapore, 138632. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from the Singapore laboratory regularly obtained whole human blood samples from healthy volunteers. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

MA: Writing – original draft, Investigation, Formal analysis. AH: Writing – review & editing. ZW: Supervision, Methodology, Writing – review & editing, Investigation. JC: Resources, Writing – review & editing, Supervision. NW: Writing – original draft, Funding acquisition, Conceptualization, 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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