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Women in infectious agents and disease 2023

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Women in infectious agents and disease: 2023

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Editorial: Women in infectious agents and disease: 2023

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Editorial on the Research Topic
Women in infectious agents and disease: 2023

A significant gender gap remains in all fields of science, with women publishing and patenting less than men (Mairesse and Pezzoni, 2015; Huang et al., 2020). Additionally, women are less likely to receive an authorship for their contribution to the research (Welle, 2022). Studies have also reported that women's research is cited less compared to that of males (Huang et al., 2020; Madsen et al., 2022). As Huang et al. (2020) stated, it is not only the total number of publications but also the percentage of women's work citations is 30% less compared to that of men. This may greatly affect women's career paths, potentially prompting some to exit the field of science.

To promote the women contribution to science we launched the Research Topic entitled as *Women in infectious agents and disease: 2023*. Seven original articles and two reviews were published on this Research Topic.

The original article by Zhou et al. aimed to conduct the molecular analysis of CAMP-negative *Streptococcus agalactiae* strains. Colonization of the birth canal, a distal reproductive system structure, is the primary mechanism of GBS transmission to a neonate (Miselli et al., 2022), leading to potential severe healthcare problems such as sepsis, meningitis, and pneumonia (Heath and Jardine, 2014). The authors stated that CAMP-negative isolates comprised 7.9% of all GBS isolates. All CAMP-negative strains were missing *cfb* gene coding for CAMP. The authors assert that these findings diverge from earlier observations, where not all CAMP-negative strains lacked the *cfb* gene (Guo et al., 2019; Tickler et al., 2019). This high frequency of gene depletion in CAMP-negative GBS was attributed to using two sets of PCR primers, which could make these results more accurate. There was no correlation between the CAMP negativity and antibiotic resistance.

Kumalo et al. analyzed the abundance of GBS in rectal and vaginal samples from pregnant women in Ethiopia. The prevalence of GBS in collected samples was 24%, falling within the globe range (Arain et al., 2015; Kwatra et al., 2016; Nishihara et al., 2017). The authors indicated no association between GBS colonization and socio-demographic data. However, there was a positive correlation between a college or above level of education and GBS colonization. An important observation was finding the high number of isolates resistant to tetracycline, ciprofloxacin, and clindamycin. The authors emphasize the importance of screening pregnant women for GBS and performing antibiotic susceptibility tests prior to selecting treatment.

Several studies have shown that a disturbed vaginal microbiome could be a risk factor for cervical cancer. To address this hypothesis, Frąszczak et al. sought to analyze the distribution of *Lactobacillus* spp. in women with abnormal Pap smear results in controls among Polish women. They found that *Lactobacillus* spp. did not differ between vaginal smear samples. However, *L. acidophilus* and *L. fermentum* were more frequent in samples collected from women in rural areas compared to urban areas. Interestingly, there were no differences in *Lactobacillus* spp. among HPV-positive and negative patients and in patients with bacterial infection. The analysis revealed a positive correlation between *L. delbrueckii* and *L. gasseri*, as well as a negative correlation between *L. fermentum* and *L. plantarum* in patients with abnormal Pap smear results.

Genital schistosomiasis is a vector-borne neglected tropical disease often diagnosed in tropical countries (Colley et al., 2014). In a study by Rausche et al., the awareness of schistosomiasis among the risk population was analyzed in Madagascar. There was higher awareness of schistosomiasis among HCWs (53.8%), while it was lower within the general population of women (11.3%). Also, the lowest awareness was among young (18–25 years old) and older (45+ years old) compared to other age groups. Family members were the primary source of knowledge among women. The authors emphasize the necessity of raising awareness about schistosomiasis among women to control this neglected tropical disease.

Tuberculosis (TB) remains a serious healthcare concern in many countries (WHO, 2013). Pathogenesis of TB includes macrophages serving as a primary site of microbial persistence (Cumming et al., 2018) which is achieved by utilizing nutrient resources such as carbohydrates, amino acids, and lipids as well as modulation of metabolic pathways favoring *Mycobacterium tuberculosis* (Mtb) propagation within the cell (Beste et al., 2013; Cumming et al., 2018; Borah et al., 2019). Slater et al. analyzed the intracellular carbon metabolic fluxes in Mtb-infected macrophages. An increased glycolytic flux toward pyruvate synthesis and reduced pentose phosphate pathway were found in infected macrophages compared to controls. The TCA pathway was inhibited in Mtb-infected THP-1 cells. Infected cells exhibited decreased levels of serine, glycine, and cysteine, while experiencing increased synthesis fluxes for aspartate, glutamine, and glutamate in macrophages. The authors state that identified metabolic changes in Mtb-infected macrophages could be targeted for developing novel therapeutics for TB.

The study by Wu et al. was aimed to analyze the role of SR2 in the pathogenesis of *Toxoplasma gondii*. Using the CRISPR-Cas9 gene editing approach, the authors identified and functionally characterized SR2, revealing its localization in the nucleus and expression only in the tachyzoite and bradyzoite stages. Additionally, the authors demonstrated that the deletion of SR2 in the type I RH strain and type II Pru strain of *T. gondii* had a limited effect on growth and bradyzoite differentiation. The disruption of this gene resulted in attenuation of the microbial virulence. The authors state that SR2 plays a role in the pathogenicity of *T. gondii* and could be a promising target for novel therapeutics against toxoplasmosis.

Chronic venous ulcer of the lower limb is a complication developed in patients with advanced venous disease (Stanek et al., 2023). *Staphylococcus aureus* is the most frequently identified microflora in patients with chronic venous ulcers (Gajda et al., 2021). However, our knowledge of virulence and resistance of strains from venous ulcer patients is limited. In the present study, Mihai et al. aimed to characterize the phenotypic virulence profiles of *S. aureus* isolated from chronic skin wounds and complete the correlation analysis with clinical presentation. The most common bacterial species was *S. aureus* capable developing a biofilm and producing toxins. The authors suggest that early analysis of bacteria linked to chronic ulcers could aid in tailoring personalized treatments for the disease.

Two review papers were published in this Research Topic.

In the first review, Patel and Rawat summarize the current knowledge on *S. aureus* MRSA pathogenesis. The authors state that biofilm formation is essential in the pathogenesis of MRSA. Biofilm formation is maintained by the expression of polysaccharide intercellular adhesin, extracellular DNA, teichoic acids, and capsule and virulence factors. These virulence factors are transcriptionally regulated by accessory gene regulator (*agr*) and *S. aureus* exoprotein expression (*sae*) locus. *Agr* regulates quorum sensing, increases virulence factor secretion, and contributes to MRSA pathogenesis *in vivo* (Bunce et al., 1992). These virulence factors could also contribute to the evasion of the immune response by this microbe. This modulation of virulence factors expression is a genetic regulatory "see-saw" of *S. aureus* pathogenesis.

In the second review, Wojciechowska et al. focused on the importance of fungi in the microbiome of neonates in the intensive care unit. The foremost important source of neonatal microbiome is that of the mother: endometrial, vaginal, gastrointestinal, and oral (Mueller et al., 2015; Yao et al., 2021). Maternal microbiome could be affected by genetics, diet, medications, infections, and stress (Cahana and Iraqi, 2020; Patangia et al., 2022; Galley et al., 2023). Additionally, the microbiome of neonates could be affected by gestation age at birth and breast milk biota (Boudry et al., 2021; Arboleya et al., 2022). The authors state that most of the research focuses on bacterial components of neonatal microbiome. However, changes in fungi species are often neglected. Studies on fungi in neonatal microbiome are urgently needed as 13% of gut microbes are fungi (Schei et al., 2017). Fungal infections remain a leading cause of morbidity and mortality in preterm neonates (Hsieh et al., 2012). The authors address the current gaps in our understanding of the role of fungi in disturbed neonatal microbiome. The importance of personalized medicine is acknowledged in this review as one of the approaches for the treatment of fungal infections in neonates.

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SK: Conceptualization, Writing – original draft. ZC: Formal analysis, Writing – review & editing. NA-M: Writing – review & editing, Software. AH: Validation, Writing – review & editing.

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Analysis of molecular characteristics of CAMP-negative *Streptococcus agalactiae* strains

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Background: *Streptococcus agalactiae* can produce CAMP factor, which can promote the β-hemolysin activity of *Staphylococcus aureus*, forming an arrow-shaped hemolysis enhancement zone at the intersection of the two bacterial species on a blood agar plate. This characteristic feature of *Streptococcus agalactiae* has led to the widespread use of the CAMP test as an identification method.

Methods: Vaginal/rectal swabs, collected from women at 35–37 weeks of pregnancy, were first inoculated into a selective enrichment broth media, then subcultured onto GBS chromogenic agar and 5% sheep blood agar sequentially. The VITEK-2 automatic identification system and MALDI-TOF MS were initially employed for identification, followed by the CAMP test. CAMP-negative strains underwent 16S rDNA and *cfb* gene sequence analysis, as well as bacterial multilocus sequence typing.

Results: A total of 190 strains were isolated, with 15 identified as CAMP-negative. Further 16S rDNA gene sequence analysis confirmed that all 15 strains were *Streptococcus agalactiae*. The MLST typing assay revealed that these 15 strains were of the ST862 type. The *cfb* gene was amplified and electrophoresed, but no specific fragments were found, indicating that these strains lack the CAMP factor due to *cfb* gene deletion. Antibiotic susceptibility tests demonstrated no resistance to penicillin, ampicillin, vancomycin and linezolid among the GBS strains. However, there are significant differences in resistance rates to tetracycline.

Conclusion: This study found that 7.9% of GBS strains isolated from the vagina/rectum of pregnant women were CAMP-negative, suggesting that the CAMP test method or primers targeting the *cfb* gene should not be used as the sole presumptive test for GBS identification.

KEYWORDS

CAMP, MLST, *Streptococcus agalactiae*, CFB, GBS

1. Introduction

Streptococcus agalactiae, also known as group B *Streptococcus* (GBS), is a Gram-positive coccus commonly found in the gastrointestinal and genitourinary tracts. Research indicates that approximately 11 to 35% of pregnant women are colonized by GBS in the vagina or rectum. (Russell et al., 2017; Bogiel et al., 2021). GBS is the primary pathogen responsible for neonatal

infections, with mother-to-child transmission being the predominant mode of infection. Roughly 50% of GBS-colonized pregnant women transmit the bacteria to their newborns. In the absence of intrapartum antibiotic prophylaxis (IAP), 1%~2% of newborns may develop sepsis, meningitis, pneumonia, or other serious complications, potentially leading to neonatal death or neurological sequelae (Puopolo et al., 2019; Geteneh et al., 2020). Since the early 1990s in the United States, GBS screening and IAP have effectively reduced the incidence of neonatal infections caused by GBS (Schrag et al., 2002; Nanduri et al., 2019).

CAMP factor is the primary virulence factor of GBS. The CAMP reaction was initially characterized as the synergistic lysis of sheep red blood cells by *Staphylococcus aureus* sphingomyelinase and CAMP factor (Christie et al., 1944). For an extended period, the CAMP phenomenon has served as a crucial basis for the laboratory diagnosis of GBS (Jin et al., 2018). Concurrently, the gene encoding CAMP factor, the *cfb* gene, has been utilized as the target of PCR assays for GBS diagnosis (Carrillo-Avila et al., 2018).

Although the CAMP test is a vital identification method for GBS, instances of GBS with negative CAMP tests have been reported occasionally (Guo et al., 2019). In this study, we employed GBS chromogenic agar plates to screen GBS from perinatal pregnant women, which demonstrated higher sensitivity compared to blood agar plates. We identified 15 strains of CAMP-negative GBS. Our laboratory has conducted preliminary molecular characteristics analyses of these strains, and the findings are presented as follows:

2. Materials and methods

2.1. Strain collection and identification

A total of 1,391 vaginal/rectal swabs were collected from pregnant women at 35–37 weeks of gestation between April 2020 and March 2021 at Pingshan General Hospital of Southern Medical University. To enhance detection rates, the vaginal/rectal swabs were inoculated into a selective enrichment broth media (Jiangmen Kailin Trading Co., Ltd., China) and incubated for 18–24 h at 35–37°C in 5% CO_2 conditions. Subsequently, the samples were subcultured onto GBS chromogenic agar plates (Zhengzhou Antu Biological Engineering Co., Ltd., China) for approximately 24 h. The purple colonies were then selected and cultivated on 5% sheep blood agar (Guangzhou Dijing Microbial Science and Technology, China) for another 24 h. Suspected isolates were initially identified using the VITEK-2 automatic identification system (BioMérieux, France) and confirmed by MALDI-TOF MS (BioMérieux, France).

2.2. CAMP test

The CAMP reactions of isolates identified as *Streptococcus agalactiae* were assessed on 5% sheep blood agar (Guangzhou Dijing Microbial Science and Technology, China) following conventional methods (Guo et al., 2019), *Streptococcus agalactiae* (ATCC13813) was employed as a positive control, *Enterococcus faecalis* (ATCC29212) as a negative control, and *Staphylococcus aureus* (ATCC25923) for the production of β-hemolysin.

2.3. 16S rDNA and cfb gene sequencing

Genomic DNA was isolated and purified using the bacterial genomic extraction kit DP302 (TIANGEN Biotech, Beijing, China). The 16S rDNA gene and *cfb* gene were amplified using Applied Biosystems 7,500 (Thermo Fisher, Foster City, USA) with primers as reported (Hongoh et al., 2003; Cezarino et al., 2008). Additionally, we designed another set of primers targeting the *cfb* gene for further verification (Forward: 5′-TGGTAGTCGTGTAGAAGCCTTA-3′; Reverse: 5′-TCCAACAGCATGTGTGATTGC-3′). All amplified fragments were analyzed by agarose gel electrophoresis and sent to Shanghai Personalbio Technology for sequencing. The obtained sequences were blasted in NCBI database. *Streptococcus agalactiae* (ATCC13813) was used as a positive control in the assay.

2.4. Multilocus sequence typing

Seven housekeeping genes of GBS (*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK* and *tkt*) were amplified separately with primers reported (Jones et al., 2003). The amplified products were analyzed by agarose gel electrophoresis and sent to Shanghai Personalbio Technology for sequencing. The obtained sequences were submitted to the MLST analysis website[1] to obtain allele numbers and STs.

2.5. Antibiotic susceptibility testing

VITEK-2 susceptibility testing, including penicillin, ampicillin, clindamycin, erythromycin, levofloxacin, tetracycline, linezolid and vancomycin, was conducted following the manufacturer's instructions using the AST-GP67 card. The results obtained after a maximum of 15 h of incubation were analyzed and interpreted by AES 7.01 software. *Staphylococcus aureus* (ATCC29213) and *Enterococcus faecalis* (ATCC29212) were used as quality control strains to ensure the credibility of the results. The D-zone test with erythromycin and clindamycin (OXOID, United Kingdom) placed at 12 mm (edge to edge) distance was performed on 5% sheep blood MH agar (Guangzhou Dijing Microbial Science and Technology, China) and incubated for 20–24 h at 37°C.

2.6. Statistical analysis

The data were reported as numbers (percentages) and compared using the chi-square test. p values <0.05 were considered statistically significant. All data were analyzed using the statistical software SPSS 19.0.

3. Results

3.1. Multilocus sequence typing

A total of 190 *Streptococcus agalactiae* strains were identified using the VITEK-2 and MALDI-TOF MS systems. Among these, 15 isolates

1 http://pubmlst.org/sagalactiae/

were found to be CAMP-negative strains (Figure 1), representing 7.9% of the total strains. Further confirmation of these strains as *Streptococcus agalactiae* was achieved through 16S rDNA sequence blast (data uploaded to NCBI GenBank: OQ680135, OQ680134, OQ680136, OQ680133, OQ680132, OQ680130, OQ680128, OQ680127, OQ680129, OQ680125, OQ680126, OQ680014, OQ680017, OQ680013, OQ680016). The 15 strains were then subjected to MLST typing, revealing that all of them belonged to ST862 (*adhP* = 16, *pheS* = 1, *atr* = 4, *glnA* = 70, *sdhA* = 9, *glcK* = 3, *tkt* = 2). The *cfb* gene was amplified using two sets of primers, one targeting the upstream region (Figure 2A, Red) and the other targeting the downstream region (Figure 2A, Blue), Agarose gel electrophoresis results indicated chromosomal deletions of the *cfb* gene in these 15 strains (Figures 2B,C), the sequence of positive control for the *cfb* gene was uploaded to NCBI GenBank: OQ871565 and OQ693682.

3.2. Antibiotic susceptibility

No GBS resistance to penicillin, ampicillin, vancomycin and linezolid was detected. The resistance rates of the 190 GBS strains to erythromycin, clindamycin, tetracycline and levofloxacin were 62.63, 56.32, 82.11 and 16.84%, respectively. Among these, the resistance rates of CAMP-positive GBS to erythromycin, clindamycin, tetracycline and levofloxacin were 63.43, 56.57, 85.14 and 18.29%, respectively. In contrast, the resistance rates of CAMP-negative GBS were 53.33, 53.33, 46.67 and 0% for erythromycin, clindamycin, tetracycline, and levofloxacin, respectively. No significant difference was observed in resistance rates for erythromycin, clindamycin and levofloxacin; however, a statistically significant difference was found for tetracycline resistance ($p < 0.01$). The results are presented in Table 1.

4. Discussion

GBS can cause miscarriage, premature delivery, and premature rupture of membranes through ascending infections in the birth canal of pregnant women. It can also lead to neonatal sepsis and meningitis through vertical transmission between mother and child. Consequently, GBS is a pathogenic bacterium that requires close monitoring during the perinatal period (Simonsen et al., 2014).

Christie et al. first reported in 1944 that the CAMP factor exhibits high specificity for GBS (Christie et al., 1944). In 1979, Bernheimer et al. isolated and purified the CAMP factor (Bernheimer et al., 1979), and its coding gene, *cfb*, was discovered in 1994 (Podbielski et al., 1994). Subsequent studies revealed that almost all GBS strains contain the *cfb* gene encoding the CAMP factor. However, some research reports identified CAMP-negative phenotypes (Hassan et al., 2002). The CAMP-negative phenotype in GBS strains with the *cfb* gene may result from transcription defects, low gene expression, or low CAMP factor activity (Podbielski et al., 1994). Between 2012 and 2018, Tickler et al. collected 31 GBS strains from 12 laboratories in the United States and Ireland, which contained deletions in or near the chromosomal region encoding the hemolysin gene *cfb*, but only 5 strains lacked the complete *cfb* gene (Tickler et al., 2019). CAMP-negative GBS has also been identified in China, Guo et al. isolated 4 CAMP-negative strains from 22 GBS strains, but only 1 strain had a *cfb* gene deficiency (Guo et al., 2019). In this study, we used two sets of primers targeting the upstream and downstream regions of the *cfb* gene and ultimately identified 15 *cfb*-deficient GBS strains from 190 isolated GBS strains, with a deficiency rate of 7.9% (15/190). This finding contrasts with the current understanding that the vast majority of GBS (>98%) contain the *cfb* gene and express the CAMP factor (Jorgensen et al., 2015).

Since most GBS strains contain the *cfb* gene, many laboratories and companies use the *cfb* gene as a target for primer design and PCR detection of GBS in vaginal/rectal swabs from pregnant women during the perinatal period (Goudarzi et al., 2015; Tanaka et al., 2016; Ferreira et al., 2018). However, our results indicate that using a GBS detection kit designed for the *cfb* gene in this region may lead to missed detections. The recently developed Xpert GBS LB XC test targets two unique GBS genes: glucosyl transferase family gene and *LysR* family gene, exhibiting higher sensitivity and specificity compared to traditional methods (Thwe et al., 2022).

The MLST results of the 15 CAMP-negative strains showed that they all belonged to the ST862 type. This type has been previously reported by Cheng et al. in Guangzhou, South China. However, the

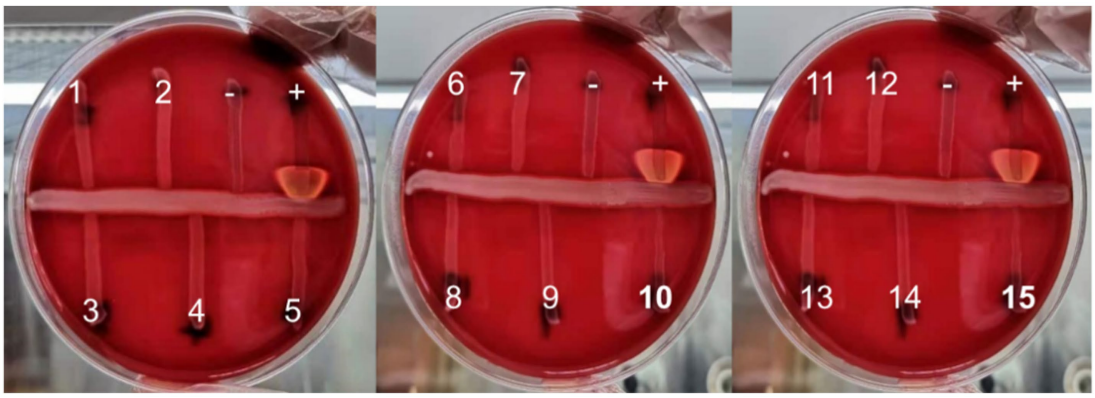

FIGURE 1
The CAMP test of 15 suspected CAMP-negative isolates of *Streptococcus agalactiae* (+: positive control, –: negative control).

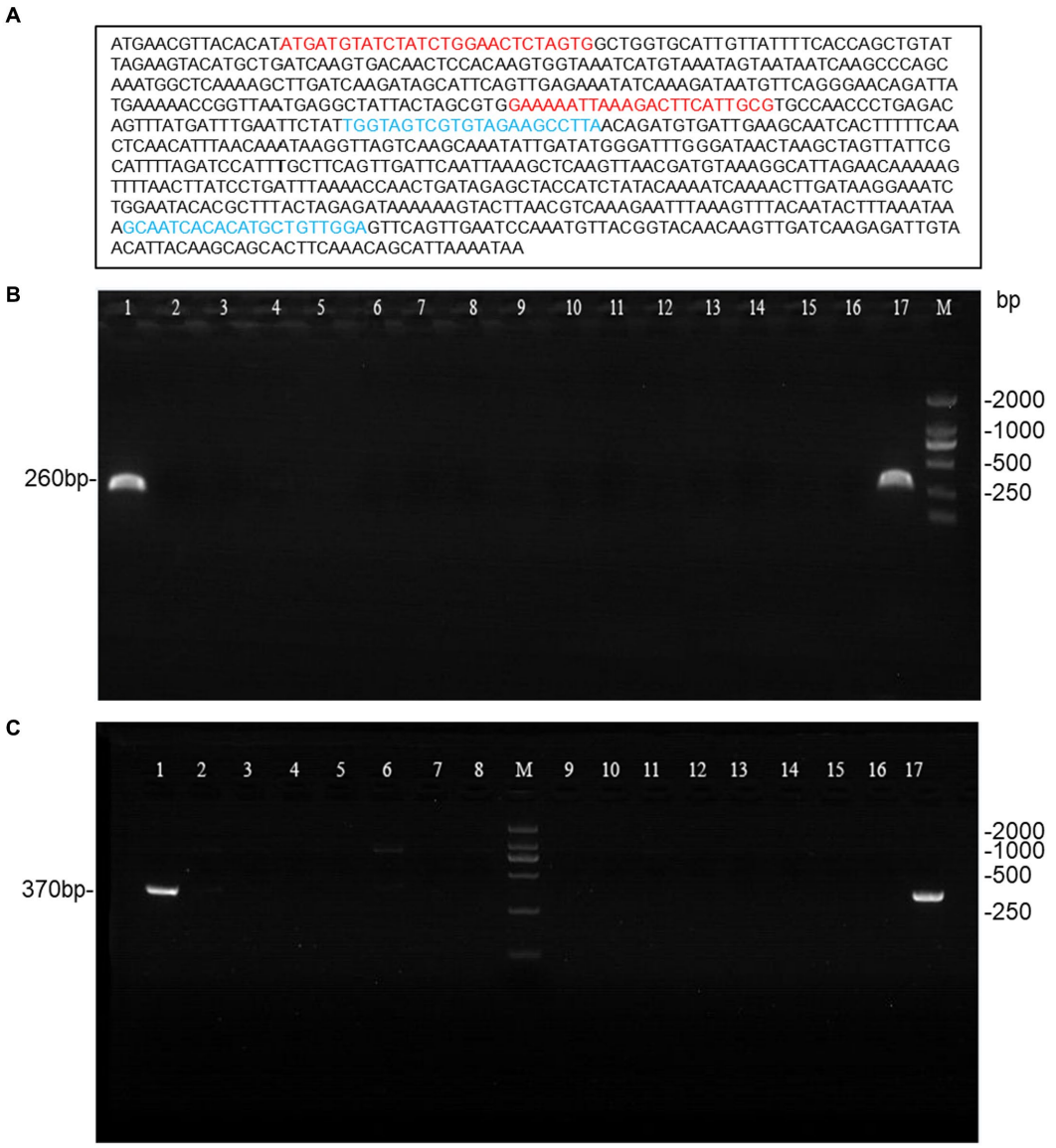

FIGURE 2
Agarose gel electrophoresis of the amplified *cfb* gene from 15 CAMP-negative isolates. **(A)** Primer design for the *cfb* gene, with red primers targeting the upstream region and blue primers targeting the downstream region. **(B,C)** The *cfb* gene amplified with the two sets primers (lane1 and 17 were positive control; lane2-16 were 15 CAMP-negative isolates; M: DL2000 DNA Marker).

six ST862 GBS strains they identified all carried the *cfb* gene (Cheng et al., 2020), contrasting with our findings. We speculate that this discrepancy may be due to local clonal expansion. The reason for the loss of the *cfb* gene requires further investigation.

Although the resistance rates of CAMP-negative GBS and CAMP-positive GBS to erythromycin and clindamycin were not statistically significant, the resistance rate of CAMP-negative GBS to tetracycline was much lower than that of CAMP-positive GBS. Whether the absence of the *cfb* gene affects GBS drug resistance warrants further study.

The CAMP factor is a secreted protein with perforating properties, known to weaken the host's immune function during systemic infection (Kurosawa et al., 2016). *In vivo* experiments have demonstrated that the CAMP factor can contribute to, or even cause, animal death, leading to the belief that it is an essential pathogenic factor (Rajagopal, 2009). However, Hensler et al. (2008) conducted *in vitro* and *in vivo* experimental studies after allelic replacement of the *cfb* gene and concluded that the CAMP factor is not necessary for GBS virulence. Therefore, there are still controversies regarding the role and mechanism of the CAMP factor in the infection process. The pathogenicity of these *cfb*-deficient GBS strains isolated in this study requires further investigation.

5. Conclusion

Based on the results of this study, 7.9% of GBS isolated from the vagina/rectum of pregnant women were CAMP-negative. As a result, the

TABLE 1 Comparison of antibiotic resistance rates between CAMP-positive and CAMP-negative GBS strains.

Antibiotic	CAMP-positive GBS		CAMP-negative GBS		$\chi 2$	*p*-value
	n/ Total	Rate (%)	n/ Total	Rate (%)		
Penicillin	0/175	0	0/15	0	–	–
Tetracycline	149/175	85.14	7/15	46.67	13.921	0.000
Erythromycin	111/175	63.43	8/15	53.33	0.602	0.438
Clindamycin	99/175	56.57	8/15	53.33	0.059	0.808
Vancomycin	0/175	0	0/15	0	–	–
Ampicillin	0/175	0	0/15	0	–	–
Levofloxacin	32/175	18.29	0/15	0	2.122	0.145
Linezolid	0/175	0	0/15	0	–	–

n, number of antibiotic resistant strains.

CAMP test should not be solely relied upon as a presumptive method for GBS identification. Utilizing primers targeting the *cfb* gene could lead to missed detections of GBS, and thus, alternative or multitarget approaches are warranted to ensure optimal diagnostic accuracy.

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 below: NCBI - OQ680135, OQ680134, OQ680136, OQ680133, OQ680132, OQ680130, OQ680128, OQ680127, OQ680129, OQ680125, OQ680126, OQ680014, OQ680017, OQ680013 and OQ680016.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Pingshan District People's Hospital of Shenzhen. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JZ, TF, and SY designed the experiments. JZ, LZ, HL, KX, YZ, and BZ executed the experiments. JZ, LZ, TF, and SY conducted the data analysis. KX and BZ collected/provided the clinical samples and information. JZ and SY wrote the manuscript with input from all of the other authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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A genetic regulatory see-saw of biofilm and virulence in MRSA pathogenesis

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Staphylococcus aureus is one of the most common opportunistic human pathogens causing several infectious diseases. Ever since the emergence of the first methicillin-resistant *Staphylococcus aureus* (MRSA) strain decades back, the organism has been a major cause of hospital-acquired infections (HA-MRSA). The spread of this pathogen across the community led to the emergence of a more virulent subtype of the strain, i.e., Community acquired Methicillin resistant *Staphylococcus aureus* (CA-MRSA). Hence, WHO has declared *Staphylococcus aureus* as a high-priority pathogen. MRSA pathogenesis is remarkable because of the ability of this "superbug" to form robust biofilm both *in vivo* and *in vitro* by the formation of polysaccharide intercellular adhesin (PIA), extracellular DNA (eDNA), wall teichoic acids (WTAs), and capsule (CP), which are major components that impart stability to a biofilm. On the other hand, secretion of a diverse array of virulence factors such as hemolysins, leukotoxins, enterotoxins, and Protein A regulated by *agr* and *sae* two-component systems (TCS) aids in combating host immune response. The up- and downregulation of adhesion genes involved in biofilm formation and genes responsible for synthesizing virulence factors during different stages of infection act as a genetic regulatory see-saw in the pathogenesis of MRSA. This review provides insight into the evolution and pathogenesis of MRSA infections with a focus on genetic regulation of biofilm formation and virulence factors secretion.

KEYWORDS

methicillin-resistant *Staphylococcus aureus* (MRSA), pathogenesis, biofilm, virulence, two-component system (TCS)

1. Introduction

Staphylococcus aureus is a normal microflora of the human nasal cavity and skin but may cause diseases such as skin and soft tissue infections, endocarditis, osteomyelitis, bacteremia, and lethal pneumonia on disruption of mucosal and cutaneous barriers due to surgical procedures, wounds, or chronic skin conditions. It is considered as one of the most common opportunistic human pathogens among immunocompromised patients, children, elderly, and patients with medical devices. Penicillin was extensively prescribed by doctors to cure *S. aureus* infections until Penicillin-resistant *S. aureus* was reported in the 1950's. To overcome this, in 1959 scientists developed a semisynthetic penicillin named methicillin which was resistant to penicillinase (Guo et al., 2020). Only after 2 years of the introduction of methicillin for clinical use, i.e., in 1961, a British scientist named Jevons isolated the first methicillin-resistant *Staphylococcus aureus* (MRSA) strain in Europe. MRSA possessed a gene *mecA* which was responsible for encoding the penicillin-binding protein 2a or 2′ (PBP2a or PBP2′). This gene was integrated into the chromosomal element (SCCmec) of methicillin-sensitive *Staphylococcus aureus* (MSSA). Since then, MRSA has been identified as one of the most notorious human pathogens across the world (Guo et al., 2020).

It is still debatable whether MRSA is more virulent than MSSA. Meta-analysis results of some of the epidemiological studies have indicated increased mortality and/or morbidity in the case of nosocomial MRSA infections such as those associated with surgery, pneumonia, and bloodstream, etc. as compared to MSSA infections (Cosgrove et al., 2003; Engemann et al., 2003; Gastmeier et al., 2005; Reed et al., 2005). Increased mortality by MRSA bacteremia as compared to MRSA pneumonia has been reported by Shurland et al. (2007). However, other studies reported no significant difference in mortality associated with nosocomial MRSA bacteremia (Cosgrove et al., 2005) or ventilator-associated pneumoniae (Zahar et al., 2005) as compared to those caused by MSSA. Another study comparing CA-MSSA and CA-MRSA skin infections also did not report any serious outcomes caused by the latter (Miller et al., 2007). To date, no study provides clear evidence that MRSA is more virulent than MSSA; however, treatment of invasive MRSA infections is challenging due to the lack of antibiotics and increased treatment costs (Gordon and Lowy, 2008).

Initially, MRSA was only associated with hospital outbreaks, more commonly termed as hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA). It was found to be responsible for 20–80% of secondary hospital infections (Krishnamurthy et al., 2014; Kemung et al., 2018). *agr* system, responsible for producing toxins for exhibiting virulence, is generally found to be either less active or mutated in HA-MRSA strains leading to high-level expression of adhesins, which aids in robust biofilm formation on implanted medical devices and are also more resistant to antibiotics (Painter et al., 2014; Suzuki et al., 2015).

Approximately 20 years after the first reported case of MRSA, community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) was reported in Detroit, Michigan, USA in 1980 (Saravolatz et al., 1982). According to the Center for Disease Control and Prevention (CDC), CA-MRSA can be defined as an infection from an MRSA culture isolated within 48 h of admission of a patient in a hospital with no previous history of hospital admission or medical treatment with surgical procedures (Gorwitz et al., 2006). According to a CDC report of 2016, two out of 100 people are carriers of CA-MRSA, suggesting its ability to spread more easily than HA-MRSA. The presence of more mobile genetic elements *viz.*, novel SCCmec elements, Panton-Valentine Leukocidin (PVL)-encoding genes, and ACME (arginine catabolite mobile element) in CA-MRSA is considered to be the plausible cause of more virulence observed in it as compared to HA-MRSA (Udo and Boswihi, 2017; Boswihi and Udo, 2018).

Recently, the evidence of the spread of MRSA from animals to humans led to the conclusion that animals are reservoirs as well as potent carriers of MRSA, which is a serious threat to human health (Voss et al., 2005; Loncaric et al., 2013; Van Boeckel et al., 2015). The plausible reason for the emergence of live-stock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is the overuse of antibiotics for increasing the yield in live-stock production (Van Boeckel et al., 2015).

2. Pathogenesis of MRSA

The entry of *S. aureus* into the endothelial tissues of the host begins when the bacterium, from an external source or from indigenous microflora, gains access to the bloodstream or underlying tissues leading to infections (Figure 1).

2.1. Mechanism of pathogenesis

2.1.1. Colonization

The colonization of the mammalian cell's extracellular matrix proteins (fibrinogen, fibronectin, elastin, collagen, laminin, and vitronectin) occurs covalently or non-covalently, mediated by several molecules of *S. aureus* that are collectively termed as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs; Table 1). Teichoic acids (TAs), which are common components of Gram-positive bacterial cell wall, also contribute significantly to the adherence to host cells (Qin et al., 2007). Approximately 15% of individuals (persistent carriers) have been reported to have continuous *S. aureus* colonization, whereas 70% of individuals have intermittent colonization (frequent *S. aureus* infection but immediate eradication), and the remaining 15% of individuals are non-carriers (Eriksen et al., 1995).

Apart from the host- and pathogen-associated factors, various other factors also play roles in *S. aureus* colonization. The nasal cavity is one of the most important sites of colonization for *S. aureus* as nose-picking can lead to the spread of bacteria to other body parts as well as to other hosts (Von Eiff et al., 2001; Wertheim et al., 2004, 2006). The nasal microbiota (*Corynebacterium* spp., *Propionibacterium acnes*, *Staphylococcus epidermis*, and *Staphylococcus lugdunensis*) and the invading pathogen *S. aureus* compete among themselves in various ways for colonization. Nutrition is one of the major limiting factors for colonization in the human nose and therefore *S. aureus* has been found to better adapt than coagulase-negative staphylococci due to the ability of the former to thrive in the low-nutrient environment (Lemon et al., 2010; Krismer et al., 2014; Liu et al., 2015; Lee et al., 2018).

The growth of *S. aureus* including MRSA has been found to be inhibited by the indigenous nasal bacterium *Staphylococcus lugdunensis* both *in vitro* and *in vivo* due to the production of an antimicrobial compound called lugdunin that can rapidly breakdown bacterial energy resources (Zipperer et al., 2016). The risk of colonization of *S. aureus* has been reported to be 6-fold lower in humans with *Staphylococcus lugdunensis* in their nasal cavities. However, nasal colonization with *Staphylococcus lugdunensis* has been reported in only 9–26% of the general population (Kaspar et al., 2016; Zipperer et al., 2016; Lee et al., 2018). *S. aureus* has been reported to compete with other commensal bacteria by the activation of host defense *via* upregulation of the production of antimicrobial proteins that are less harmful to it than to others (Krismer et al., 2017; Lee et al., 2018). Apart from the nasal cavity, other sites in the human body where *S. aureus* colonizes are armpits (8%), abdomen (15%), intestine (17–31%), perineum (22%), vagina (5%), and pharynx (4–64%; González-García et al., 2023). The

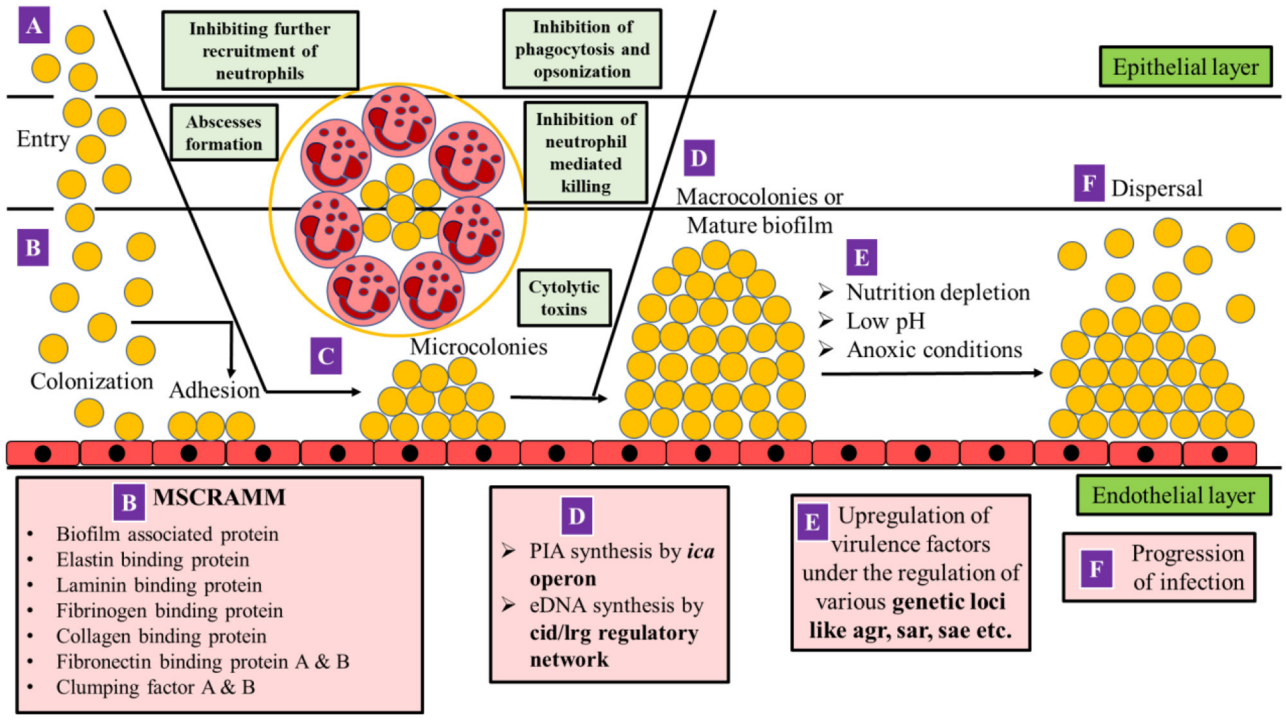

FIGURE 1
Mechanism of pathogenesis in MRSA. **(A)** *S. aureus* gains access to the bloodstream or underlying tissues due to a breach in cutaneous or mucosal barriers. **(B)** Colonization occurs on the endothelial layer by adhesion with the help of MSCRAMM. **(C)** As the *S. aureus* cells multiply, polymorphonuclear leukocytes (PMNs) are recruited, and a fibrin pseudo-capsule termed as abscess is formed with the help of coagulase proteins secreted by *S. aureus* which surrounds bacterial cells and recruited (PMNs). *S. aureus* can inhibit the further entry of PMNs at the site of infection, opsonization, phagocytosis, and neutrophil-mediated killing. The bacterium which is already phagocytosed by PMNs releases cytolytic toxins such as hemolysins, leukocidins such as Panton-Valentine Leukocidin (PVL) and PSM peptides to escape the host defense. The bacterial cells further multiply leading to the formation of microcolonies on the endothelial layer. **(D)** Synthesis of poly intracellular adhesin (PIA) and extracellular DNA (eDNA) is initiated by the *ica* operon and *cid/lrg* regulatory network, respectively, resulting in the formation of macrocolonies or a mature biofilm. **(E)** As biofilm matures, the core becomes anoxic, nutrient-deprived, and acidic in pH which triggers the upregulation of various virulence factors (*agr*, *sar*, and *sae*), which mediates **(F)** dispersal of biofilm as well as the progression of infection (adapted from Moormeier and Bayles, 2017; Sedarat and Taylor-Robinson, 2022).

virulence genes responsible for toxin production in *S. aureus* are downregulated during the colonization process.

The multiplication of bacteria after colonization on both biotic and abiotic surfaces leads to the formation of a three-dimensional complex community of bacteria within a layer of exopolysaccharide (EPS) termed as "biofilm" (Figure 1; Costerton et al., 1999; Sedarat and Taylor-Robinson, 2022). The accumulation of biofilm is facilitated by the formation of microcolonies due to the production of exopolysaccharide (a major component of biofilm), which comprises 97% water and 3% of structural and functional molecules (Guzmán-Soto et al., 2021). EPS of biofilm contains both positively and negatively charged groups as well as hydrophobic groups. The negatively charged groups of EPS include phosphates, sulfates, carboxyl groups, glutamic acid, and aspartic acid, whereas the positively charged groups include amino sugars such as polysaccharide intercellular adhesin (PIA). Despite positively charged PIA being a major component, the overall charge on the EPS surface is negative, serving as a better target site for positively charged drug candidates (Idrees et al., 2021).

The major EPS produced by *S. aureus* is polysaccharide intercellular adhesin (PIA), also known as poly-*N*-acetyl-glucosamine (PNAG; Mack et al., 1996). PIA/PNAG has a net positive charge and it promotes intercellular interactions by binding to the negatively charged surfaces of bacterial cells such as teichoic acids (O'Gara, 2007; Vergara-Irigaray et al., 2008). The multivalent electrostatic interaction of the cationic PIA polymer with the negatively charged wall teichoic acids on staphylococcal cells as revealed by single-cell force spectroscopy confirmed that the cationic nature of PIA is crucial for its attachment to the cell surface and intercellular adhesion (Formosa-Dague et al., 2016). PIA mutants exhibited a decreased ability to adhere to each other (Peng et al., 2022). There is no evidence for a covalent linkage of PIA to the cell surface (Cue et al., 2012). PIA is evident for biofilm formation under high-shear flow conditions such as those found inside catheters as compared to low-shear conditions such as those in subcutaneously implanted tissue, ocular infections, or platelet concentrate (Nguyen et al., 2020).

In addition to PIA/PNAG, biofilms contain bacterial proteins, eDNA, ions, and carbohydrates (Guzmán-Soto et al., 2021) as essential components with their ratios being variable. A number of staphylococcal strains exhibit PIA/PNAG-independent biofilm formation where the secreted proteins and extracellular DNA substitute for PIA/PNAG (Cue et al., 2012).

TABLE 1 Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs).

Adhesion protein	Encoding gene	Function	References
Biofilm-associated protein	*bap*	Promotes the formation of biofilm	Cucarella et al., 2001
Elastin-binding protein	*ebpS*	Promotes the binding of soluble elastin peptides and tropo- elastin to *S. aureus* cells, however, it is not able to promote bacterial adherence to immobilized elastin and, therefore, is not a microbial surface component recognizing adhesive matrix molecule (MSCRAMM)	Park et al., 1991; Roche et al., 2004
Laminin-binding protein	*eno*	Binds to laminin by destructing extracellular matrix on the host cell leading to invasion and dissemination	Carneiro et al., 2004
Fibrinogen-binding protein	*fib*	Interacts with alpha chain of fibrinogen and its derivative, fibrin, and causes repression of fibrinogen-dependent platelet aggregation	Palma et al., 2001
Collagen-binding protein	*cna*	Facilitates bacterial adherence to collagenous tissues such as cartilage of host cell	Patti et al., 1993
Fibronectin-binding protein A	*fnbA*	Promotes bacterial attachment to both soluble and immobilized forms of fibrinogen (Fg) by means of a unique binding site localized within the 17 C-terminal residues of the gamma-chain of human Fg. Both plasma proteins (Fn and Fg) function as a bridge between the bacterium and host cell	Wann et al., 2000
Fibronectin-binding protein B	*fnbB*	Multifunctional protein which promotes bacterial attachment to fibrinogen, elastin, and fibronectin	Roche et al., 2004; Burke et al., 2011; Pietrocola et al., 2016
Clumping factor A	*clfA*	Promotes bacterial attachment to the gamma-chain of human fibrinogen making it a dominant factor responsible for human platelet aggregation	Siboo et al., 2001
Clumping factor B	*clfB*	Promotes bacterial attachment to both alpha- and beta-chains of human fibrinogen, mediates bacterial attachment to the highly keratinized squamous epithelial cells from the nasal cavity *via* interaction with cytokeratin K10 (K10)	Ní Eidhin et al., 1998; O'Brien et al., 2002

Extracellular DNA (eDNA) is another important structural component of biofilm matrix serving as a "glue" for the community due to its adhesive property. The destabilization of a matured biofilm by using DNAase I in *Pseudomonas aerugionosa* reported by Whitchurch et al. (2002) suggested the role of eDNA in biofilm formation in pathogenic bacteria. In a matured biofilm when there is a scarcity of nutrients, a subsequent population of damaged cells is eliminated in order to release nutrients for healthy cells. This self-destructive or suicidal act of cells is termed as autolysis or programmed cell death (PCD; Lewis, 2000). Along with the nutrients, the lysed cells also release genomic DNA in the form of eDNA. Rice et al. (2007) reported that only a small fraction of the bacterial population (<1%) within the biofilm undergoes cell lysis to release a sufficient amount of eDNA required for biofilm stability (Bayles, 2007). eDNA can also confer antibiotic resistance mainly due to the horizontal gene transfer of eDNA to the healthy cells of biofilm (Molin and Tolker-Nielsen, 2003; Tetz et al., 2009).

Capsular polysaccharides (CPs) are long-chain polysaccharides attached covalently to the peptidoglycan layer of the cell wall. They aid in the colonization of the host as well as in biofilm formation, hence are indirectly involved in the progression of invasive diseases. However, the contrasting effect on the virulence of *S. aureus* is observed by the presence or absence of a capsule depending upon the type of infection (O'Riordan and Lee, 2004; Tuchscherr et al., 2010). CP enhances virulence in murine models of bacteremia (Thakker et al., 1998; Watts et al., 2005), septic arthritis (Nilsson et al., 1997), abscess formation (Portols et al., 2001), and surgical wound infection (McLoughlin et al., 2006). On the contrary, in mammary gland infections (Tuchscherr et al., 2005) and in catheter-induced endocarditis (Baddour et al., 1992; Nemeth and Lee, 1995), CP mutants are more virulent. This is likely because CP also inhibits the adherence of the underlying adhesins to their specific target molecule (Phlmann-Dietze et al., 2000; Risley et al., 2007). CP-negative *S. aureus* strains are frequently isolated from patients with osteomyelitis, mastitis, or cystic fibrosis, providing evidence that the loss of CP expression (due to mutations in any of the genes essential for CP biosynthesis or in the promoter region; Cocchiaro et al., 2006; Tuchscherr et al., 2010) may be advantageous for *S. aureus* during chronic infection (Herbert et al., 1997; Lattar et al., 2009; Tuchscherr et al., 2010). CPs also help bacteria in evading the phagocytic uptake by the host immune system and also protect the important bacterial cell wall constituents (Berni et al., 2020). Out of 13 serotypes of *S. aureus* (Berni et al., 2020), serotypes 1 and 2 (rarely reported among clinical isolates) produce mucoid colonies while the remaining serotypes produce non-mucoid colonies on a solid medium. Serotypes 5 and 8 (prevalent among clinical isolates as well as commensal sources) constitute ∼25 and 50%, respectively, of the isolates recovered from humans from various geographic locations of the world (O'Riordan and Lee, 2004).

Teichoic acids are one of the major components of Gram-positive bacterial cell wall. These diverse anionic carbohydrate-based polymers are categorized into two classes: (1) lipoteichoic acids (LTAs), which are embedded in the lipid bilayer with the help of a diacylglycerol lipid anchor that can extend from the cell surface to the peptidoglycan layer, and (2) wall teichoic acids (WTAs), which are covalently attached to peptidoglycan matrix *via* a phosphodiester linkage to the C6 hydroxyl of the N-acetyl muramic acid sugars and can extend through and beyond the cell wall (Swoboda et al., 2010; Berni et al., 2020). WTAs comprise ∼60% of the total cell mass of Gram-positive bacteria. *S. aureus* WTAs are essential for adhesion to host tissues as well as to artificial surfaces

including glass and polystyrene (Gross et al., 2001). WTAs null mutants are defective in their ability to produce biofilm; however, the reduced production of PNAG (an inevitable component of biofilm) was not reported, suggesting an independent role of WTAs in biofilm formation (Vergara-Irigaray et al., 2008). WTA mutant *S. aureus* strains were also unable to adhere and colonize the nasal epithelial cells as well as endothelial tissues of the kidney and the spleen derived from cotton rats. D-alanylation, which takes place on LTA, was reported to be intact on these strains, implying that WTAs were independently involved in cell adhesion and eventually biofilm formation (Weidenmaier et al., 2005). The involvement of WTAs in host colonization, infection, and biofilm formation makes it an important virulence factor and the enzymes involved in its biosynthesis can be novel targets for the discovery of new antimicrobials (Weidenmaier and Peschel, 2008).

Signaling molecules such as cyclic AMP (cAMP) are not only linked with the regulation of carbon metabolism and stringent response but also with the expression of virulence genes and biofilm formation in Gram-positive bacteria (Schilcher and Horswill, 2020). *S. aureus* requires two molecules of ATP and the enzyme diadenylyl cyclase DacA to synthesize c-di-AMP. Phosphodiesterase GdpP is involved in the degradation of c-di-AMP (Corrigan et al., 2011). Screening for essential genes of *S. aureus* revealed that *dacA* disruption was not possible, indicating the importance of c-di-AMP formation for the viability of the bacterium (Chaudhuri et al., 2009). On the other hand, *gdpP* deletion in the *S. aureus* SEJ1 strain yielded a 3-fold increase in biofilm formation, highlighting the fact that high levels of c-di-AMP induced biofilm formation in this strain. However, similar experimental conditions did not replicate the results in *S. aureus* USA300 LAC or its corresponding *gdpP* mutant (Corrigan et al., 2011). Single nucleotide polymorphisms (SNPs) or deletions in *gdpP* in various homogenous oxacillin-resistant (HoR) *S. aureus* isolates exhibited a decrease in the expression of *icaADBC* and *agr* and an increase in the expression of penicillin-binding protein 2 (PBP2). This resulted in an absence of polysaccharide content in the biofilm and the formation of a more proteinaceous biofilm (Pozzi et al., 2012). Apart from this, *gdpP* mutants were also impaired in their ability to form eDNA suggesting that c-di-AMP is essential for eDNA release as well (DeFrancesco et al., 2017). The role of c-di-AMP in biofilm formation may be strain dependent as different studies yielded different biofilm phenotypes in *gdpP* mutants of *S. aureus* (Schilcher and Horswill, 2020).

S. aureus possesses small α-helical peptides called phenol soluble modulins (PSM) which act as surfactants and disrupt cell–cell interactions within the biofilm better than proteases, unlike other bacteria that commonly use nucleases and proteases for biofilm detachment (Otto, 2008). The detachment and dispersal of planktonic cells are mediated by changes conferred in pH, nutrition, waste accumulation, oxygen depletion, etc. (Otto, 2008).

2.1.2. Invasion and infection

Once colonization is established, the bacteria adhere and start multiplying on wounded tissues, resulting in the upregulation of virulence genes and the production of toxins that further aid in disease progression. The initially expressed adhesion genes are now downregulated (Novick, 2003; Foster et al., 2014).

During the onset of infection, 95% of iron is within host cells in the form of serum iron bound to host proteins. In such a situation of iron starvation, *S. aureus* secretes high-affinity iron-binding proteins such as staphyloferrin (Drechsel et al., 1993) and aureochelin (Courcol et al., 1997). *S. aureus* can also initiate transcription of Isd (iron surface determinants)-mediated heme-iron transport that facilitates the release of heme from hemoglobin, haptoglobin, and hemopexin. This free heme transports across the plasma membrane *via* the iron complex and free iron is released in the cytoplasm of bacterial cell as a result of oxidative degradation (Maresso and Schneewind, 2006). This helps the bacterium in survival along with evasion from host defense (Liu, 2009).

Gram-positive bacteria including *S. aureus* secrete a diverse array of chemo-attractants such as peptidoglycan (Dziarski and Gupta, 2005), N-terminal lipoylated structure of lipoproteins (Nguyen and Götz, 2016), formylated peptides (Krepel and Wang, 2019), and unmethylated CpG sequences in DNA (Hemmi et al., 2000). This gradient of chemo-attractants induces proinflammatory signaling and activation of local immune response by recruitment of neutrophils and macrophages to the site of infection *via* a process called chemotaxis (Kolaczkowska and Kubes, 2013). Moreover, PSMs secreted by *S. aureus* sheds lipoproteins that also act as neutrophil chemo-attractants (Hashimoto et al., 2006; Hanzelmann et al., 2016). These structures collectively termed as "pathogen-associated molecular patterns" (PAMPs) are specific to bacteria and hence are recognized by the host immune system which activates Toll-like receptors (TLRs; Kumar et al., 2011).

A fibrin pseudo-capsule termed as abscess is formed within 2–6 days of infection (Kobayashi et al., 2015) with the help of coagulase proteins secreted by *S. aureus* which surround bacterial cells and recruited polymorphonuclear leukocytes (PMNs). After recruitment, *S. aureus* further inhibits neutrophil extravasation, activation, and chemotaxis in various ways. The binding of the members of the staphylococcal superantigen-like protein (SSL) family *viz.* SSL2 and SSL4 to TLR2, SSL10 to CXCR4, and SSL5 to GPCRs, such as P-selectin glycoprotein ligand- 1 (PSGL-1; which normally binds to the P-selectin anchor on endothelial cells) on the leukocyte surface, subsequently blocking neutrophil adhesion and extravasation to the site of infection. SelX protein has also been reported to have a similar function as SSL5 (Cheung et al., 2021). CHIP (chemotaxis inhibitory protein) blocks neutrophil recognition of chemotactic factors while Eap (extracellular adhesion protein) prevents neutrophil binding to endothelial adhesion molecule (ICAM-1; Chavakis et al., 2002; de Haas et al., 2004). *S. aureus* protease staphopain degrades CXCR2 (which recognizes cytokines), leading to the inhibition of neutrophil migration toward cytokines (Laarman et al., 2012). Another Geh lipase has been reported that removes the pro-inflammatory lipoylated N- terminus of the bacterial lipoproteins, thereby disguising these PAMPs from neutrophils (Chen and Alonzo, 2019).

Phagocytosis is also inhibited by biofilm formation *via* PIA/PNAG production (refer Section 2.1.1), protective surface structures such as capsules (refer Section 2.1.1), and aggregation.

The combined non-redundant activity of coagulase and von Willebrand factor-binding protein (vWbp) of *S. aureus* produces a protein called thrombi which binds to prothrombin (factor II of the coagulation process) and forms a complex called staphylothrombin (Friedrich et al., 2003; Cheng et al., 2010). In the absence of a vascular damage signal, staphylothrombin cleaves fibrinogen from the host cells and forms fibrin clots. Fibrinogen-binding proteins of *S. aureus* such as clumping factor A (ClfA) bind to these clots resulting in the formation of fibrin-containing bacterial aggregates (McAdow et al., 2011). FnBPA and FnBPB can also activate the aggregation of platelets (Fitzgerald et al., 2006). *S. aureus* cells that are already phagocytosed by PMNs fight for their survival by releasing cytolytic toxins which cause pore formation and eventually cell lysis (Figure 1; Peschel and Sahl, 2006; Chambers and DeLeo, 2009; Liu, 2009; Löffler et al., 2010; Spaan et al., 2013; Stapels et al., 2014).

The expression of the capsule, clumping factor A, Protein A, and various other complement inhibitors on the cell surface of the bacterium may help to overcome opsonophagocytosis, i.e., the marking of the bacterial pathogen by antibodies (immunoglobulins, Igs) or complement factors for efficient phagocytosis. The Igs bind to phagocytes by the F_c region and to the pathogen by the F_{ab} region. The presence of Igs on the bacterial surface not only marks it for opsonization but also activates the classical pathway of complement fixation (Cheung et al., 2021). *S. aureus* produces three proteins to overcome opsonization by antibodies. (1) Surface protein A (SpA) non-specifically binds to the F_c region of IgG (Forsgren and Sjöquist, 1966) and F_{ab} region of IgM, which acts as a B cell superantigen and causes B cell apoptosis. It also initiates the production of plasma B cells that specifically recognizes only protein A, thereby diverting the immune response away from other virulence factors (Goodyear and Silverman, 2003; Pauli et al., 2014). (2) Sbi (*S. aureus* binder of IgG) binds to the complement factor H and C3 apart from the F_c region of IgG (Zhang et al., 1998; Atkins et al., 2008). (3) SSL10 also binds to the F_c region of IgG and prevents receptor-mediated phagocytosis (Itoh et al., 2010).

All three pathways (lectin, classical, and alternative) of the complement system possess the C3 convertase which cleaves C3 into C3a and C3b. The deposition of C3b on the bacteria marks it for opsonization. Other complement factors such as C5a (formed *via* the interaction of C3-C3b) act as a chemoattractant for the recruitment of more immune cells to the site of infection (Rooijakkers et al., 2005). Staphylococcal complement inhibitor (SCIN) inhibits C3 convertase, thus reducing the C3b deposition and C5a chemoattractant formation leading to the blockage of all three pathways of the complement system. Extracellular fibrinogen binding protein (Efb) of *S. aureus* binds to the C3 component *via* the C-terminus and fibrinogen *via* its N-terminus, covering the bacteria in a fibrinogen layer that inhibits the activation of the complement system as it fails to sense the surface-bound C3b (Ko et al., 2013). A homologous protein of Efb i.e., extracellular complement binding protein (Ecb), although lacking the fibrinogen binding activity, can inhibit the C3 convertase of the alternative pathway and all C5 convertases (Jongerius et al., 2010). Some *S. aureus* proteins such as collagen adhesion protein (Cna) inhibit the classical pathway (Kang et al., 2013), SdrE protein inhibits the alternative pathway (Sharp et al., 2012; Zhang et al., 2017) and the extracellular adherence protein (Eap) inhibits both lectin and classical pathways (Woehl et al., 2014). Finally, the SSL7 protein binds to the C5 component of the complement system as well as to the F_c region of the IgA antibody, inhibiting its recognition (Langley et al., 2005).

Proteolytic activity of various *S. aureus* proteins such as staphylococcal serine protease (V8 protease; SspA), cysteine protease (SspB), metalloprotease (aureolysin; Aur), and staphopain (Scp) have also been reported to inhibit opsonization (Dubin, 2002). Though the primary function of these proteases is nutrient acquisition, they may also destroy various immune defense proteins such as aureolysin cleaves C3 (Laarman et al., 2011) and V8 protease cleaves Igs (Rousseaux et al., 1983).

Despite the several mechanisms to evade phagocytosis, neutrophils can still manage to engulf *S. aureus* cells. Primary granules of neutrophil synthesize the enzyme Myeloperoxidase (MPO) which produces reactive oxygen species (ROS) and antimicrobial peptides (AMPs) such as defensins. Secondary granules of neutrophil secrete antimicrobial proteins such as lysozyme. *S. aureus* has evolved with various mechanisms to overcome both ROS and AMPs (Cheung et al., 2021). The orange pigment that gives *S. aureus* (*aureus* stands for golden) its name, Staphyloxanthin has been reported to scavenge the free radicals originating from ROS activity (Pelz et al., 2005; Clauditz et al., 2006). *S. aureus* synthesizes the enzyme superoxide dismutase which converts superoxide to less toxic H_2O_2 (Mandell, 1975; Clements et al., 1999). Furthermore, the catalase KatA and alkyl hydroperoxide reductase C AhpC detoxify the H_2O_2 by converting it into oxygen and water (Mandell, 1975; Cosgrove et al., 2007). MPO is directly inhibited by a staphylococcal peroxidase inhibitor (SPIN; De Jong et al., 2017).

Defensins and other AMPs are usually positively charged and hence are attracted to the negatively charged cell membrane and exhibit their bactericidal activity by forming pores in the bacterial membrane (Joo and Otto, 2015). The *dlt* operon of *S. aureus* esterifies hydroxyl groups in teichoic acids with alanyl residues which imparts an extra positive charge per alanine into the bacterial cell membrane, increasing the net charge and thereby inhibiting the binding of AMPs (Peschel et al., 1999). Moreover, a membrane-bound enzyme MrpF (multiple peptide resistance factor) adds Lys-PG on the outer layer of the cell membrane which also decreases the affinity of AMP binding (Peschel et al., 2001; Ernst et al., 2015). Finally, *S. aureus* secretes an enzyme named OatA, which acetylates the muramic acid residues of peptidoglycan reducing the efficacy of the neutrophil secreted antibacterial protein lysozyme which is otherwise very effective against other Gram-positive bacteria (Bera et al., 2005).

Neutrophil extracellular traps (NETs) are considered to be an integral component of the human immune system as they play a major role in host defenses during bacterial infections. NETs consist of activated neutrophils and DNA backbone along with proteins of various biological functions. NETs can ensnare but not kill *S. aureus*. The trapped bacteria from NETs can be released by degradation of the DNA backbone *via S. aureus* DNase leading to the persistence of the chronic infection. Eap, a protein secreted by *S. aureus,* can bind and aggregate linearized DNA, hindering the

formation of NETs. Apart from this, the pathogen overcomes NET-mediated killing by expressing the surface protein FnBPB, which can neutralize the bactericidal activity of histones (Speziale and Pietrocola, 2021).

When encountered by neutrophils, *S. aureus* biofilms release the leukocidins such as LukAB and PVL (Panton-Valentine Leukocidin), which not only promotes *S. aureus* survival during phagocytosis but also induces the formation of NETs. Neutrophils and NETs can penetrate *S. aureus* biofilm but are unable to disrupt it (Malachowa et al., 2013). NETs can accumulate in organs and can cause tissue damage, as the infection progresses (Saffarzadeh et al., 2012; Weber, 2015). Therefore, the induction in the formation of NETs rather than blocking its antibacterial activity benefits the bacteria by favoring the colonization of deeper tissues, and thus providing better access to metabolic resources. This ensures the safer and optimal survival of bacteria in the host (Speziale and Pietrocola, 2021).

The abscess initially formed and matured within 6–14 days of infection accompanied by fibroblastic proliferation and tissue repair at the abscess margin and the formation of a fibrous capsule at the periphery (Kobayashi et al., 2015). The disruption of abscesses leads to the spread of *S. aureus* beneath the skin surface as well as in the bloodstream causing bacteremia. The bacterium can now adhere to endothelial surfaces and platelets causing endocarditis, metastatic abscesses, and bacterial uptake into endothelial cells where antibiotics and host defense molecules struggle to reach (Chavakis et al., 2005; Weidenmaier et al., 2005; Löffler et al., 2014). If endovascular spread cannot be controlled then systemic blood coagulation, massive production of microorganism-associated molecular pattern molecules (MAMP) and superantigen toxin-mediated cytokine storms can result in systemic inflammation, sepsis, multiple organ failure, and eventually death (Thomer et al., 2016; Lee et al., 2018).

3. Virulence factors involved in pathogenesis

S. aureus secretes a diverse array of virulence factors such as MSCRAMMs, hemolysins, leukotoxins, Protein A, exfoliative toxins, staphylococcal enterotoxins (SEs), and toxic-shock syndrome toxin-1 (TSST-1; refer Sections 3.1–3.6). Genes encoding these virulence factors (except MSCRAMMs) are located on the accessory genome, which comprises of mobile genetic elements (MGEs) such as plasmids, insertion sequences, pathogenicity islands, transposons, and prophages. These MGEs not only encode the genes for virulence factors production but also contain antibiotic-resistance determinants. Plasmids and transposons contain antibiotic-resistance genes while prophages and pathogenicity islands consist of toxins and other virulence determinants (Malachowa and DeLeo, 2010; Lindsay, 2019). The large family of staphylococcal pathogenicity islands (SaPIs) is widely known for enterotoxins and TSST. Toxins such as staphylococcal superantigen-like genes (SSLs), lipoprotein-like toxins (LPLs), α toxin, PSM peptides, leukocidin LukDE, and some enterotoxins are encoded on genomic islands and vary in their expression among different *S. aureus* isolates (Langley et al., 2010; Malachowa and DeLeo, 2010; Nguyen et al., 2015). Some other *S. aureus* toxins such as PVL, exfoliative toxins A and B, staphylokinase, immune evasion proteins such as CHIPS and SCIN, and several other enterotoxins are encoded on prophages (Cheung et al., 2021). β toxin encoding gene *hlb* has been reported to be non-functional in many *S. aureus* strains by the insertion of phage-encoding genes for CHIPS, SCIN, and staphylokinase (Carroll et al., 1993). This process is termed as "negative conversion" and it can be repaired by phage excision, which is important for infectious colonization (Katayama et al., 2013). Genomic islands such as vSAα, vSAβ, and vSAγ have also been reported to encode a diverse array of virulence factors. These MGEs have lost their ability to be transferred by non-MGE-specific mode of transfer and hence are now very stable and in fact are the characteristic of the entire species (Kläui et al., 2019). The genes encoding MSCRAMMs are located on the core genome, which is evident as they also exhibit general functions in the commensal lifestyle of *S. aureus* (Cheung et al., 2021).

3.1. MSCRAMMs

S. aureus MSCRAMMs can bind to various proteins present in the extracellular matrix (ECM) of the host. Fibronectin-binding proteins, FnbPA and FnbPB, are responsible for bacterial attachment to fibronectin *in vitro* as well as binding to foreign bodies and plasma clots (Nizet and Bradley, 2011). Fibrinogen binding proteins or clumping factors, i.e., ClfA and ClfB (Ní Eidhin et al., 1998; O'Connell et al., 1998), are responsible for firm attachment of *S. aureus* to vascular thrombi in the situation of flow stress within the bloodstream (Fowler et al., 2000). Endocarditis studies in rats have reported reduced virulence in ClfA mutant *S. aureus* strains (Moreillon et al., 1995). Collagen-binding protein Cna is responsible for adherence to collagenous tissues such as cartilages (Patti et al., 1992). A Cna null mutant strain of *S. aureus* showed attenuated virulence in a murine septic arthritis model (Patti et al., 1994). Other important MSCRAMMs have been discussed in Table 1.

3.2. Hemolysins

Hemolysins are toxins that lyse red blood cells as well as immune cells by binding to their specific receptors. Major classes of hemolysins include α, β, and γ hemolysins which are under the regulation of *agr* and *sae* locus. δ hemolysin (under the regulation of *agr* locus), also classified as Phenol Soluble Modulins (PSM), does not require a receptor to exhibit its hemolytic activity (Kong et al., 2016). α hemolysin/alpha toxin, encoded by the *hla* gene, is one of the major toxins under *agr* regulation. The alpha toxin, a 319 amino acid long pore-forming toxin, is shaped like a beta-barrel that can bind to disintegrin and metalloprotease 10 (ADAM10) receptor present on the cell membrane of the human host cell. α toxin can lyse red blood cells and leukocytes but is unable to neutralize neutrophils (Valeva et al., 1997). In animal models, *hla* mutant strains have been reported to cause less disease severity than the wild-type strains, suggesting the importance of

hla toxin in staphylococcal infections (Wilke and Wardenburg, 2010; Berube and Bubeck Wardenburg, 2013). β hemolysin is a non-pore-forming toxin that can hydrolyze sphingomyelin, lyse erythrocytes (at low temperature), and monocytes but not lymphocytes and granulocytes (Walev et al., 1996). Its mode of action is still unclear, but it has been hypothesized that as the toxin acts as a sphingomyelinase, it may destabilize the bilipid layer of the plasma membrane of host cells, leading to an alteration in plasma membrane fluidity (Vandenesch et al., 2012). γ hemolysin is a bi-component toxin consisting of two polypeptide chains namely S (slow, HlgA, or HlgC) and F (fast, HlgB). The F component binds to the phosphatidylcholine of host cells, whereas the S component causes cell lysis by binding to the cell membrane of host cells (Meyer et al., 2009). Vandenesch et al. (2012) have reported the lysis of rabbit RBC as well as leukocytes such as macrophages, neutrophils, monocytes, and granulocytes by γ hemolysin. δ hemolysin/phenol soluble modulins (PSMs) are the only peptide toxins of *S. aureus* whose expression is under the direct control of AgrA and independent of RNAIII (Queck et al., 2008). δ toxin is small, amphipathic, and possesses a high affinity to lipids (Vandenesch et al., 2012). These multifunctional peptides produced by many *S. aureus* strains are hemolytic to erythrocytes, various organelles, bacterial protoplasts, and spheroplasts (Verdon et al., 2009). PSMα is a strong pro-inflammatory toxin that can lyse neutrophils post-phagocytosis and also contributes majorly to biofilm formation (Otto, 2014).

3.3. Leukotoxins/leukocidins

Leukotoxins such as LukDE, LukGH (LukAB), and Panton-Valentine Leukocidin (PVL) are pore-forming toxins under the regulation of *sae* and *agr* locus (Queck et al., 2008; Cheung et al., 2011; Alonzo and Torres, 2014). Alonzo et al. (2013) have reported lysis of phagocytic cells such as macrophages, dendritic cells, and lymphocytes by binding of LukDE toxin on the CCR5 chemokine receptor present in these immune cells. LukGH (LukAB), similar to PSMα peptide, binds to CD11b receptor in humans and causes lysis of immune cells after phagocytosis (DuMont et al., 2013). Panton-Valentine Leukocidin (PVL), commonly found in community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA), is a two-component toxin (LukS and LukF proteins that binds to TLR2 and TLR4, respectively, in animal models, and C5aR and C5L2 in humans) responsible for causing pore formation in the leukocyte cell membrane, which eventually results into cell lysis and tissue necrosis followed by skin and soft tissue infections (SSTIs; Rasigade et al., 2010). PVL has been reported to be 100-fold more potent than other leukotoxins (Kong et al., 2016).

3.4. Protein A

Protein A (*spa*) is one of the major proteases among the *agr* downregulated surface proteins (Cheung et al., 2011). It can activate the TNFα receptor (TNFR1) in the lung parenchyma, resulting in a pro-inflammatory response (Gómez et al., 2004). Once released from the *S. aureus* envelope, Protein A can combat the host's humoral immune response by blocking Fc receptor-mediated opsonophagocytosis and may trigger apoptosis (refer Section 2.1.2; Peterson et al., 1977; Goodyear et al., 2006; Pauli et al., 2014; Le and Otto, 2015).

3.5. Exfoliative toxins (ETs)

ETs are serine proteases (under the regulation of *agr* locus) that cleaves the protein desmoglein 1, which leads to the disruption of desmosomal cell linkages causing the detachment of skin epidermis, which results in a surge of infection (Eyre and Stanley, 1987; Hanakawa et al., 2002). ETs are also superantigens but less potent compared to other superantigens such as TSST-1 (Monday et al., 1999). ETs are responsible for a disease named staphylococcal scaled skin syndrome (SSSS), which majorly infects neonates and infants; however, adults who are immunocompromised or have renal impairment are also prone to it. The major symptoms include blistering of the skin, loss of superficial skin layers, dehydration, and other secondary infections (Bukowski et al., 2010).

3.6. Staphylococcal enterotoxins (SEs) and toxic-shock syndrome toxin-1 (TSST-1)

Staphylococcal enterotoxins (SEs), secreted by entero-toxigenic *S. aureus* strains, are one of the most common causes of foodborne diseases as these toxins are heat stable. Based on antigenic heterogeneity, there are more than 24 different SEs identified that are under the regulation of *agr* and *sae* locus (Grispoldi et al., 2021). SEA to SEE, SEG to SEI, and SER to SET have reported emetic activity, while staphylococcal-like (SE*l*) proteins (SE*l*L and SE*l*Q) are not emetic in animal models, whereas SE*l*J, SE*l*K, SE*l*M to SE*l*P, SE*l*U, SE*l*U2, and SE*l*V are yet to be tested (Argudín et al., 2010). Ses induce cytokine release by T-cell activation and proliferation causing cell death *via* apoptosis and toxic shock syndrome (Balaban and Rasooly, 2000; Lin et al., 2010). SEF has been renamed as toxic shock syndrome toxin (TSST-1), widely known as a superantigen that can bind to HLA class II molecule on antigen-presenting cells and on T cell receptors leading to massive T-cell activation, proliferation, and release of cytokines (referred to as "cytokine storm") *viz*., IL-8, MIP-3, IL-2, and TNFα inducing apoptosis and cell death due to lethal toxic shock. In lethal cases, TSST can lead to severe shock, organ dysfunction, and eventually death (Fraser and Proft, 2008; Otto, 2014; Stach et al., 2014). An epidemic was reported in the US from 2004 to 2014, which was found to be common among women using high-absorbency tampons in menstruation (mTSS). However, the incidence rate of mTSS was significantly reduced after the change in manufacturing and awareness regarding the use of tampons (Sharma et al., 2018). Currently, around 50% of TSS infections (such as skin and soft tissue infections) are due to non-menstrual toxic shock syndrome (nmTSS; Sharma et al., 2019).

4. Genetic regulation of pathogenesis

The pathogenesis of *S. aureus* is largely dependent on the formation of biofilm and the production of virulence factors. The genetic regulatory network of biofilm and virulence in *S. aureus* is quite complex including a cross-regulation between different components *viz.* MSCRAMMs, *ica* locus, *cid/lrg* network, *tar* genes, *cap* operon, codY, SarA, and *agr* and *sae* TCS (Figure 2). During the onset of infection, the genetic systems responsible for initial colonization (*viz.* MSCRAMMs genes) and biofilm formation (*viz., ica* locus, *cid/lrg* network, and *tar* genes) are upregulated facilitating adherence to host tissue. As the infection progresses, there is a scarcity of nutrition and oxygen in the matured biofilm (due to an increase in bacterial cell density) that relieves the repression mediated by CodY (a global transcriptional regulator in *S. aureus* which represses the virulence genes with respect to nutrient availability and metabolism). Consequently, there is an upregulation of genetic systems responsible for the production of toxins (*agr* and *sae* TCS) that aid in acquiring nutrition, evading immune cells, and spread of infection. This suggests that apart from regulatory proteins, environmental factors such as nutrition, oxygen, pH, and reactive oxygen species (ROS) can also play a significant role. In this instance, the adhesion factors expressed initially are downregulated leading to the dispersal of biofilm. The dispersed planktonic cells can now adhere and colonize to other sites and spread the disease. The up- and downregulation of adhesion genes involved in biofilm formation and genes responsible for synthesizing virulence factors during different stages of infection act as a genetic regulatory see-saw in the pathogenesis of MRSA (Figure 3).

4.1. Regulation of biofilm formation

4.1.1. PIA/PNAG production

The structural proteins, namely IcaA, IcaD, IcaB, and IcaC encoded by the *ica* operon synthesize PIA/PNAG. IcaA and IcaD are transmembrane proteins that simultaneously work as N-acetylglucosaminyltransferase-converting NAG monomers to PNAG oligomers of <20 residues in length. The membrane-bound IcaC protein transports the PNAG oligomers across the cell membrane. Bacterial cell surface-associated IcaB protein deacetylates the PIA/PNAG oligomers that impart a positive charge to them and thus facilitates binding with the negatively charged bacterial cell surface (Cue et al., 2012).

4.1.1.1. Regulation of *ica* locus

The expression of the *icaADBC* operon is regulated by various direct and indirect factors. SarA and σ_B are global regulatory proteins of the operon, whereas some local proteins such as IcaR and TcaR regulate comparatively fewer genes. IcaR directly regulates the *icaADBC* operon, whereas proteins such as Rbf and Spx have an indirect effect (Cue et al., 2012; Figure 4).

(i) **IcaR**

IcaR repressor gene (which encodes the IcaR protein) is transcribed in a divergent manner from the other four genes (Conlon et al., 2002). The binding of IcaR to a DNA region immediately 5′ to the *icaA* gene and deletion of the short nucleotide sequence of 5 bp (TATTT) within the *icaA-icaR* intergenic region could significantly increase the expression of the *icaADBC*. *icaR* deletion can cause a 100-fold increase in *ica* locus expression and a 10-fold increase in PNAG/PIA production (Jefferson et al., 2004).

(ii) **Sar A**

SarA is a global regulatory protein as it affects the expression of many pathogenic genes in *S. aureus,* hence making it a major virulence factor. SarA protein can function as both activator and repressor of transcription (Bayer et al., 1996; Beenken et al., 2003; Weidenmaier et al., 2005; Oscarsson et al., 2006). SarA can directly bind to the *ica* promoter, enhancing PIA/PNAG production and, subsequently, biofilm formation (Valle et al., 2003; Jefferson et al., 2004; Tormo et al., 2005). The most important role that SarA plays in promoting biofilm formation is the repression of extracellular proteases and nucleases (Schilcher and Horswill, 2020). An elevated expression of proteases was reported in *sarA* mutants (Mrak et al., 2012; Zielinska et al., 2012), which also exhibited a decreased affinity for fibronectin binding (Blevins et al., 2002) and an inability to form static or flow cell biofilm (Beenken et al., 2003). Inhibition of all three classes of proteinases i.e., serine, cysteine, and metalloprotease (Tsang et al., 2008), or concurrent mutation of four extracellular proteases i.e., Aur, ScpA, SspA, and SspB can restore biofilm formation in *sarA* mutants (Loughran et al., 2014).

(iii) **TcaR**

TcaR is a member of the MarR family of transcription factors of *S. aureus*. Jefferson et al. (2004) demonstrated, *via* northern analysis, a 5-fold increase in transcription of the *ica* locus by the inactivation of the *tcaR* gene. The bacterial adherence and PIA/PNAG production were found to increase up to 500-fold in the *icaR tcaR* double mutant, indicating that *tcaR* is a negative regulator of the *ica* locus.

(iv) **σ_B**

σ_B is an alternative sigma factor found in *S. aureus* and other Gram-positive bacteria which is activated by signal transduction in response to environmental stress such as high temperature, extreme pH, high osmolarity, and use of antibiotics (Donegan and Cheung, 2009). Rachid et al. (2000) reported the role of σ_B in biofilm formation under high salinity and osmotic stress in an *S. aureus* mucosal isolate. σ_B has been reported to have a role in the expression of *ica* operon. Valle et al. (2003) reported that *sarA*- $\sigma_B$double mutants showed a decrease in *icaADBC* expression, but, on the other hand, also showed a significant increase in PIA/PNAG production and biofilm formation as compared to single *sarA* mutants. These results suggested the role of σ_B in the upregulation of a factor directly involved in the turnover of PIA/PNAG. An enhancement in PIA-dependent biofilm formation has been reported in σ_B mutants due to an increased accumulation of IcaC protein (Valle et al., 2019).

The available literature on σ_B-mediated regulation of PIA-dependent biofilm formation is contradictory. Cerca et al. (2008) studied *icaADBC* and *icaR* expressions in σ_B mutant SA113 and Newman strains of *S. aureus*. They concluded that σ_B was a positive

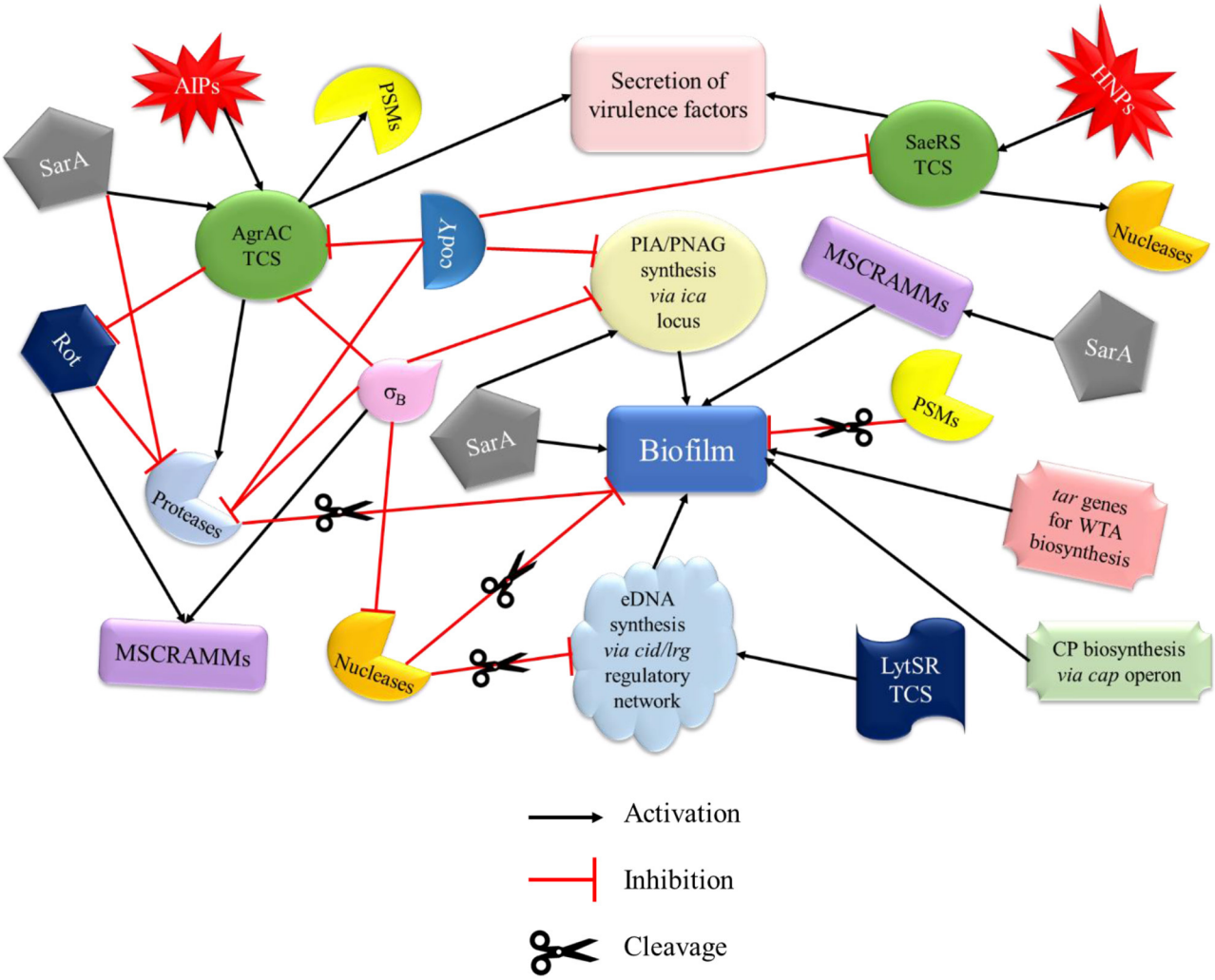

FIGURE 2
Regulatory network of biofilm and virulence in MRSA. The regulation of biofilm formation and virulence factors of MRSA involves a cross-talk of various systems that drive its pathogenesis. MSCRAMMs, PIA synthesis, CP, WTA biosynthesis genes, and SarA promote biofilm formation. PSMs and proteases (activated by AgrAC TCS) are directly involved in the disruption of biofilm, whereas nucleases (activated by SaeRS TCS and involved in the cleavage of eDNA), σ_B, and codY (by inhibiting PIA synthesis) play an indirect role. Agr and Sae TCSs, which are mainly involved in the secretion of virulence factors of MRSA, are primarily activated by AIP and HNP, respectively. AgrAC TCS can also be activated by SarA and repressed by σ_B and codY. codY is also involved in the downregulation of SaeRS TCS. MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; PIA, polysaccharide intercellular adhesin; CP, capsule; WTA, wall teichoic acid; PSMs, phenol soluble modulins; AIP, autoinducing peptides; HNP, human neutrophil peptides; Rot, repressor of toxins.

regulator of both *icaADBC* and *icaR* transcripts. IcaR has been reported to be a weak repressor of *icaADBC* in *S. aureus* strains. Rachid et al. (2000) reported a decrease in the transcription of the *ica* locus in the *sigB* mutant of the MSSA MA12 strain. A biofilm-negative phenotype of the *sigB* mutant SH1000 and USA300 strain LAC (CA-MRSA isolate) was found to have no significant changes in the *ica*- dependent PIA formation but showed an enhanced Agr and protease activity (Lauderdale et al., 2009). However, both these strains were capable of forming PIA-independent biofilms (Boles and Horswill, 2008; Pozzi et al., 2012; Atwood et al., 2015). These contradictory results may be due to variations in some strain-specific properties, and the use of different media as well as biofilm models used in these studies (Schilcher and Horswill, 2020).

(v) **Rbf**

Rbf (regulator of biofilm), a member of the AraC/XylS family, is a transcriptional regulatory protein that plays a critical role in biofilm formation in *S. aureus* (Lim et al., 2004). Rbf positively regulates *icaADBC* transcription by inhibiting *icaR* expression (Gallegos et al., 1997). Cue et al. (2009) analyzed whether the activation of *icaADBC* mediated by Rbf is through direct binding to *ica* promoter or by repression of *icaR*. The results indicated that Rbf was unable to directly bind to the *icaADBC* promoter, suggesting that the

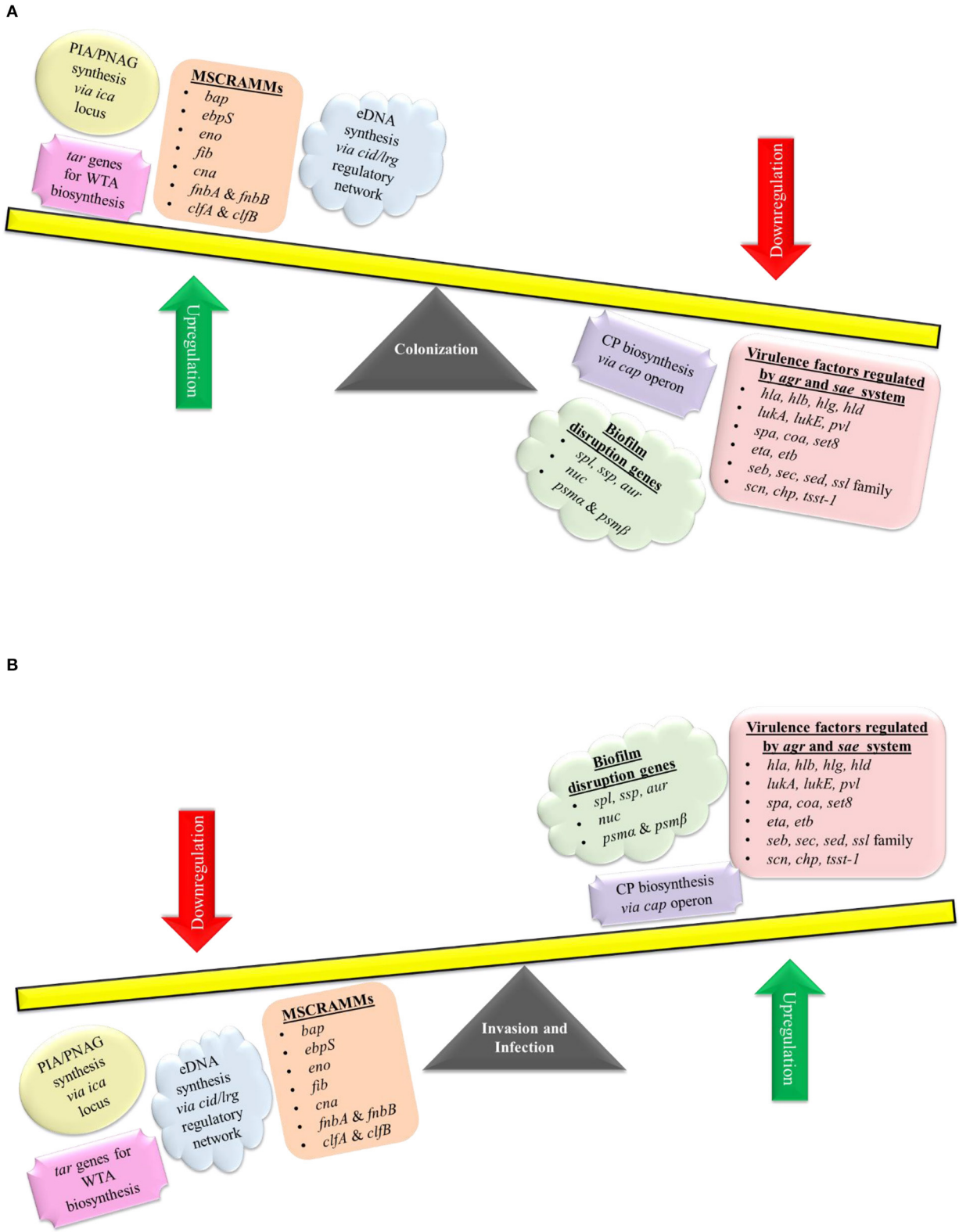

FIGURE 3
Genetic regulation of pathogenesis. *S. aureus* pathogenesis acts like a see-saw mechanism that involves an up- and downregulation of biofilm and virulence genes during different stages of infection. **(A)** For colonization, *S. aureus* upregulates *ica* locus, *cid/lrg* network, *tar* genes, and MSCRAMMs genes (*bap*, *ebpS*, *eno*, *fib*, *cna*, *fnbA*, *fnbB*, *clfA*, and *clfB*), which aids in adherence to host tissues as well as biofilm formation. Genes involved in biofilm disruption and virulence factor production are downregulated during colonization. As the infection progresses there is a scarcity of nutrition and oxygen in the matured biofilm, which leads to an **(B)** upregulation of biofilm disruption genes (*spl*, *ssp*, *aur*, *nuc*, *psmα*, and *psmβ*) and genetic systems such as *cap* operon, *agr* & *sae* TCS (which regulates genes such as *hla*, *hlb*, *hlg*, *hld*, *lukA*, *lukB*, *pvl*, *spa*, *coa*, *set8*, *eta*, *etb*, *seb*, *sec*, *sed*, *ssl* family genes, *scn*, *chp*, and *tsst-1*) that aid in acquiring nutrition, evading immune cells and spread of infection. Genes involved in colonization and biofilm formation are downregulated during the invasion and infection process.

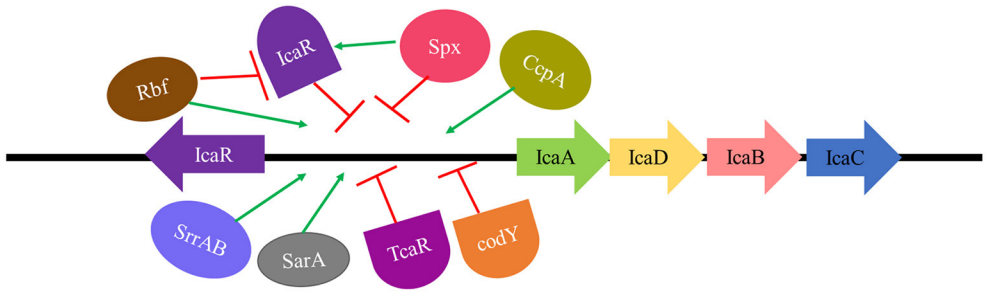

FIGURE 4
Regulation of *ica* locus. SarA, SrrAB TCS, and CcpA are direct activators, whereas Rbf exhibits an indirect effect in *ica* locus activation by repressing IcaR (repressor of *ica* locus). IcaR, TcaR, and codY are direct inhibitors whereas Spx exerts an indirect effect on *ica* locus repression by activating IcaR (repressor of *ica* locus).

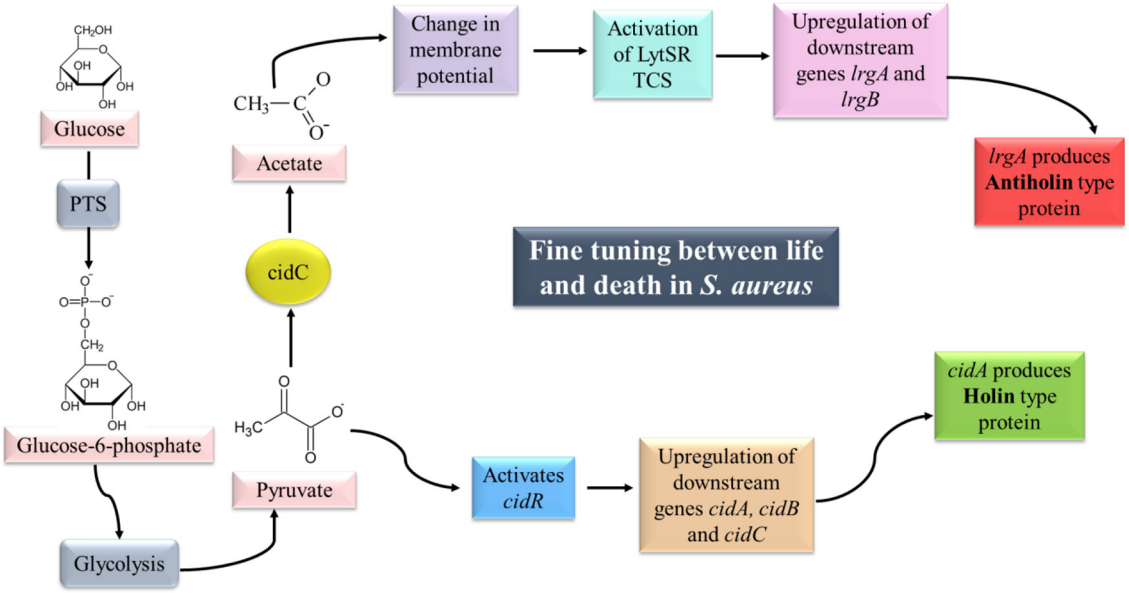

FIGURE 5
cid/lrg system and its regulation. LytSR TCS is activated by sensing a change in membrane potential (due to the conversion of pyruvate to acetate by CidC). Phosphorylated LytR results in upregulation of the *lrgAB* operon which is responsible for the production of Antiholin-like protein. Pyruvate on the other hand can activate cidR which can upregulate the *cidABC* operon, responsible for the production of Holin-like protein. Synthesis of Holin and Antiholin-like proteins maintain a fine tuning between life and death in *S. aureus* (adapted from Bayles, 2007; Sadykov and Bayles, 2012).

expression of *icaADBC* by Rbf is indirect *via* inhibition of the expression of *icaR*.

(vi) **Spx**

Spx protein is a global transcriptional regulator that can act both as an activator as well as a repressor. It blocks transcription by directly binding to the α subunit of RNA polymerase, thereby inhibiting its interactions with target genes (Nakano et al., 2010). Spx mutants were found to exhibit increased transcription of *icaADBC* along with a decrease in *icaR* transcription. Thus, it can be concluded that Spx downregulates *icaADBC* expression by upregulating *icaR* expression. However, the exact mechanism of increased expression of *icaR* by Spx is still unclear (Pamp et al., 2006).

(vii) **SrrAB**

SrrAB is a two-component system (TCS) that prevents the phagocytosis of *S. aureus* by neutrophils in anaerobic conditions which is often found in the core region of a mature biofilm. Ulrich et al. (2007) demonstrated that phosphorylated SrrA protein can bind to 100 bp upstream of the promoter of *ica* operon resulting in an increase in its expression. Under anoxic conditions, the SrrAB mutant showed downregulation of *icaA* transcription and PIA/PNAG production. However, the SrrAB mutation did not exhibit any change in *icaR* expression, suggesting the direct activation of the *icaADBC* by SrrAB.

(viii) **CcpA and CodY**

The expression of *icaADBC* in *S. aureus* is also affected by changes in the levels of the TCA cycle intermediates with respect to the metabolic state of the cell (Vuong et al., 2005). The genes encoding for enzymes involved in the TCA cycle are usually repressed by CcpA in the presence of high glucose concentration among the majority of Gram-positive bacteria including *S. aureus*. High-intracellular levels of glucose-6 phosphate and fructose 1,6

bisphosphate regulate the activity of CcpA, which has been reported to be an activator of *icaADBC*. The synthesis of branched-chain amino acids is repressed due to the downregulation of the TCA cycle in high-glucose conditions. CodY transcriptional regulatory protein, which is responsive to branched-chain amino acids and a repressor of *ica* operon, is also downregulated, resulting in further activation of *icaADBC* and enhanced biofilm formation. When the biofilm matures and nutritional scarcity arises then CcpA activity is repressed, TCA cycle and branched chain amino acid synthesis genes are upregulated, resulting in the activation of *codY* and inhibition of *icaADBC* and, subsequently, biofilm formation. Thus, CodY and CcpA are both regulators of *icaADBC* expression in *S. aureus* (Seidl et al., 2008; Fujita, 2009). Mlynek et al. (2020) suggested that PIA synthesis in cells with low codY activity majorly contributes to biofilm formation. CodY regulates PIA-dependent biofilm formation and *codY* mutant strains exhibit different biofilm phenotypes. For instance, a transposon insertion in the *codY* gene of the clinical isolate S30 revealed reduced biofilm formation and PIA production (Tu Quoc et al., 2007). On the contrary, a *codY* allelic replacement mutation in two *S. aureus* clinical isolates SA564 and UAMS-1, reported an elevated biofilm formation, probably due to higher transcription of *icaA* and increased PIA synthesis during *in vitro* growth (Majerczyk et al., 2008). However, CodY-mediated regulation of the *icaADBC* locus is independent of IcaR (Majerczyk et al., 2008), SarA, and RNAIII (Majerczyk et al., 2010).

4.1.2. cid/lrg system

cidABC and *lrgAB* operons of *S. aureus* encodes bacteriophage such as holins and antiholins, respectively (Brunskill and Bayles, 1996b). These operons have also been reported to have a crucial role in staphylococcal murein hydrolase activity (Groicher et al., 2000; Rice et al., 2003). In bacteriophages, holins are responsible for the transport of murein hydrolases across the cytoplasmic membrane for cell lysis and antiholins have an inhibitory activity on the holins (Young, 1992). Similarly, in *S. aureus* the cell lysis and eDNA release are controlled by the antagonistic activity of *cid* and *lrg* gene products. Mutations in either *cid* or *lrg* operons lead to variation in biofilm formation which suggests that fine-tuning between survival and death is essential for a robust biofilm formation (Rice et al., 2007; Mann et al., 2009).

4.1.2.1. Regulation of *cid/lrg* system

The cell death and lysis of *S. aureus* controlled by the *cid/lrg* system are under the regulation of another TCS, i.e., LytSR and transcriptional regulator cidR (Figure 5).

LytSR is a two-component regulatory system composed of a sensory histidine kinase protein (LytS) and a response regulator (LytR), which has been reported to control the expression of *lrgAB* operon (Brunskill and Bayles, 1996a; Sharma-Kuinkel et al., 2009). LytS senses the change in membrane potential of the cell which may be caused due to accumulation of acetate in the media under conditions of excessive glucose and oxygen (overflow metabolism; Rice et al., 2005; Patton et al., 2006). Activated LytS in turn phosphorylates the response regulator LytR, which can induce *lrgAB* expression. Mutation in *lytSR* genes has been reported to cause increased levels of autolysis due to increased murein hydrolase activity (Brunskill and Bayles, 1996a). Hence, LytSR-mediated control over the *lrgAB* operon is essential to regulate the autolysis phenomenon in biofilm.

cidR is a LysR type of transcriptional regulator (LTTR) responsible for DNA binding. Yang et al. (2005) reported two overlapping transcripts (*cidABC* and *cidBC*) of *cid* operon as revealed by northern blot analysis. The transcription of *cidBC* is induced by σ_B, while the transcription of *cidABC* is dependent on CidR and CcpA (Sadykov et al., 2019). The presence of excess glucose and/or acetic acid induces the transcription of *cidABC*. CidC-encoded pyruvate oxidase converts the end product of glycolysis, i.e., pyruvate into acetate (Patton et al., 2005). Pyruvate is also believed to serve as a co-inducer molecule for the activation of *cidR* (Schell, 1993).

In planktonic cells of *S. aureus*, the expression of *cidABC* is induced under excess glucose leading to a high rate of glycolysis which in turn inhibits aerobic respiration and diverts the carbon flow through fermentation pathways, known as the Crabtree effect. With enhanced murein hydrolase activity due to the activation of *cidABC*, holin-like proteins are produced that aid in the death and lysis of weakened cells. On the other hand, fermentative metabolism and CidC protein lead to the conversion of pyruvate into organic acids such as acetic acid. Acidification leads to disruption in the membrane potential of the cell which is sensed by LytSR TCS that further controls the expression of *lrgAB*. *lrgAB* transcription leads to the production of antiholin-like protein, thereby maintaining a fine balance between survival and death of *S. aureus* (Sadykov and Bayles, 2012).

The decrease in the expression of *lrgA via* induction of *sigB* by hyperglycemia-related factors such as advanced glycation end products (AGEs) secreted during diabetic foot infection (DFI) in *S. aureus* increases the release of eDNA, thereby enhancing biofilm formation (Xie et al., 2020). σ_B has also been reported to repress the expression of the secreted thermonuclease Nuc. Cell-free supernatants of a USA300 *sigB* mutant were found to inhibit the biofilm formation of different *S. aureus* strains. Subsequent fractionation and mass spectroscopy analysis revealed that Nuc was an active component in the supernatant which is responsible for the cleavage of eDNA, subsequently inhibiting biofilm formation. Kiedrowski et al. (2011) reported enhancement in biofilm formation in several *S. aureus* strains, including the USA300 lineage upon deletion of the *nuc* gene. The biofilm-negative phenotype of the *sigB* mutation has been observed to be partially repaired in a *nuc-sigB* double mutant.

4.1.3. Capsule biosynthesis

CP5 and CP8 capsules (prevalent among *S. aureus* isolates) consist of repeating units of N-acetyl mannosaminuronic acid, N-acetyl L-fucosamine, and N-acetyl D-fucosamine. *S. aureus* have different serotypes due to the difference in glycosidic linkages between the sugars and the sites of O-acetylation of the mannosaminuronic acid residues of the capsule (O'Riordan and Lee, 2004; Kuipers et al., 2016). The pathway for capsule (CP) biosynthesis occurs in the cytoplasm *via* three distinct reactions, in which the universal cell envelope substrate UDP-D-N-acetylglucosamine (UDP-D-GlcNAc) is converted into

the three different nucleotide-coupled sugars: UDP-N-acetyl-D-fucosamine (UDP-D-FucNAc), UDP-N-acetyl-L-fucosamine (UDP-L-FucNAc), and UDP-N-acetyl-D-mannosaminuronic acid (UDP-D-ManNAcA; Figure 6; Rausch et al., 2019).

The first reaction is catalyzed by the enzymes CapD and CapN that converts UDP-D-GlcNAc into the first soluble precursor UDP-D-FucNAc. The phosphosugar moiety of UDP-D-FucNAc is transferred to the membrane-anchored lipid carrier undecaprenyl-phosphate ($C_{55}P$) by CapM, yielding lipid I_{cap} (Li W. et al., 2014). The second reaction is catalyzed by the enzymes CapE, CapF, and CapG which convert UDP-D-GlcNAc into a second soluble precursor UDP-L-FucNAc. The transferase CapL further attaches L-FucNAc to lipid I_{cap}, resulting in the formation of second CP lipid intermediate, lipid II_{cap} (Kneidinger et al., 2003). The third reaction is catalyzed by the epimerase CapP and the dehydrogenase CapO, which converts UDP-D-GlcNAc into a third soluble precursor UDP-D-ManNAcA (Kiser et al., 1999; Portols et al., 2001). The transmembrane protein CapI transfers the UDP-D-ManNAcA moiety to lipid II_{cap}, generating the final capsule precursor lipid III_{cap} (Rausch et al., 2019). The modification of $C_{55}P$ coupled trisaccharide is carried out by acetyltransferase CapH, which catalyzes the O- acetylation of L-FucNAc residues at the C3 position in CP5 strains (Bhasin et al., 1998). The putative flippase CapK and the polymerase CapJ translocate the modified precursor to the outer surface of the cell membrane where polymerization occurs (Sau et al., 1997; O'Riordan and Lee, 2004). The attachment of CP precursors to the MurNAc (N-acetylmuramic acid) moiety of peptidoglycan occurs *via* an unknown mechanism that possibly involves a member of the LCP (LytR-CpsA-Psr) family of proteins (Kawai et al., 2011; Chan et al., 2014). This process has been assumed to release the lipid carrier $C_{55}P$, which enters a new synthesis cycle (Rausch et al., 2019).

4.1.3.1. Genetic regulation of capsule expression

cap5 and *cap8* are allelic gene loci that constitute a 17.5 kb region of the chromosomal DNA. Both loci consist of 16 linked genes [*cap5A (cap8A)* to *cap5P (cap8P)*], which are transcribed in one direction and are involved in biosynthesis, acetylation, transport, and regulation of capsule biosynthesis. In total, 12 out of these 16 genes are nearly identical in both loci (Sau et al., 1997; Rausch et al., 2019). The genes distinguishing CP5 and CP8 strains exhibit very little homology and are located in the central region of the loci (*cap5H*, *cap5I*, *cap5J*, and *cap5K* and similarly for *cap8*; O'Riordan and Lee, 2004). Wann et al. (1999) integrated *cap5HIJK* genes into the CP8 strain *via* homologous recombination resulting in a reciprocal loss of *cap8HIJK*. The recombinant strain started producing CP5, indicating that indeed *cap5HIJK* genes were responsible for the CP5 serotype.

cap8 and *cap5* gene expression are both positively regulated by *agr* locus as well as *sarA*. Single mutants of *agr* and *sarA* as well as *agr-sarA* double mutant studies have reported *agr* locus to be a major regulator of *cap8* gene expression. *sarA* gene was also found to be responsible for the activation of *cap8* gene expression at the transcriptional level but its effect was minor as compared to *agr* (Luong et al., 2002). Positive regulation of *cap5* gene expression by *agr* locus both *in vitro* and *in vivo* was reported in the rabbit endocarditis model. Similar to *cap8* gene regulation, *sarA* was also found to exert a lesser positive impact on *cap5* gene expression (van Wamel et al., 2002). Another global regulator *mgr* belonging to the MarR family of transcriptional regulators has been reported to upregulate CP8 biosynthesis and nuclease expression but downregulated the production of alpha toxin, protease, Protein A, and coagulase (Luong et al., 2003). The global repressor codY has also been reported to repress the *cap* operon under high-nutrient conditions, i.e., early and exponential growth phase (Pohl et al., 2009; Majerczyk et al., 2010). Phosphorylated SaeR (SaeRS TCS) binds to the promoter of the *cap* operon and represses both SigB- and SigA-dependent promoter activities (Keinhoerster et al., 2019). Environmental factors also play a major role in capsule expression both *in vitro* and *in vivo*. High-salt conditions, iron limitation, and growth on solid medium enhance the CP production, whereas high glucose, low oxygen, high CO_2, alkaline conditions, and yeast extract repress the CP production. CP was reported to rarely express during *ex vivo* analysis of bacteria recovered from cystic fibrosis patients and only a few isolates were CP positive. This might be due to the high CO_2 concentration in the lungs. Similarly, in cases of nasal colonization also, only a part of the *S. aureus* population was reported to be CP-positive (Keinhoerster et al., 2019).

4.1.4. WTAs biosynthesis

WTA biosynthesis was first characterized in *Bacillus subtilis* 168, which makes poly(glycerol) phosphate, and hence the genes involved in its synthesis were known as *tag* genes (for teichoic acid glycerol). *Bacillus subtilis* W23 and *S. aureus* make poly(ribitol) phosphate and hence the genes involved in its synthesis were known as *tar* genes (for teichoic acid ribitol; Ward, 1981; Figure 7).

The first three steps of the biosynthetic pathway are catalyzed by the enzymes TagO (TarO), TagA (TarA), and TagB (TarB), respectively. In the cytoplasm, N-acetylglucosamine phosphate (GlcNAc-1 phosphate) is transferred to an undecaprenyl phosphate (bactoprenyl phosphate) by a reversible phosphosugar transferase enzyme, TagO (TarO; Soldo et al., 2002; Price and Tsvetanova, 2007). Furthermore, TagA (TarA), an N-acetylmannosaminyl transferase, catalyzes the transfer of ManNAc from UDP-ManNAc to the C4 hydroxyl of GlcNAc residue to form ManNAc-β1,4-GlcNAc disaccharide (Ginsberg et al., 2006; Zhang et al., 2006). Finally, TagB (TarB), a glycerophosphotransferase, catalyzes the transfer of a single phosphoglycerol unit from CDP- glycerol (synthesized by TagD or TarD) to the C4 hydroxyl of ManNAc to complete the synthesis of linkage unit (Ginsberg et al., 2006; Bhavsar et al., 2007). The WTA linkage unit is highly conserved in all Gram-positive bacterial strains characterized so far. After these first three initial steps, the WTA pathways diverge (Brown et al., 2013).

In *S. aureus,* TarF (homolog of TagF that acts as a polymerase), which acts as a primase, adds one additional glycerol phosphate unit to the linkage unit (Swoboda et al., 2010). The assembly of the poly(ribitol) phosphate main chain is carried out by one of the two bifunctional poly(ribitol) phosphate primases/polymerases known as TarK/TarL. *S. aureus* TarL is a bifunctional enzyme having both primase and polymerase activities (Meredith et al., 2008). A cytidylyl transferase TarI and an alcohol dehydrogenase TarJ

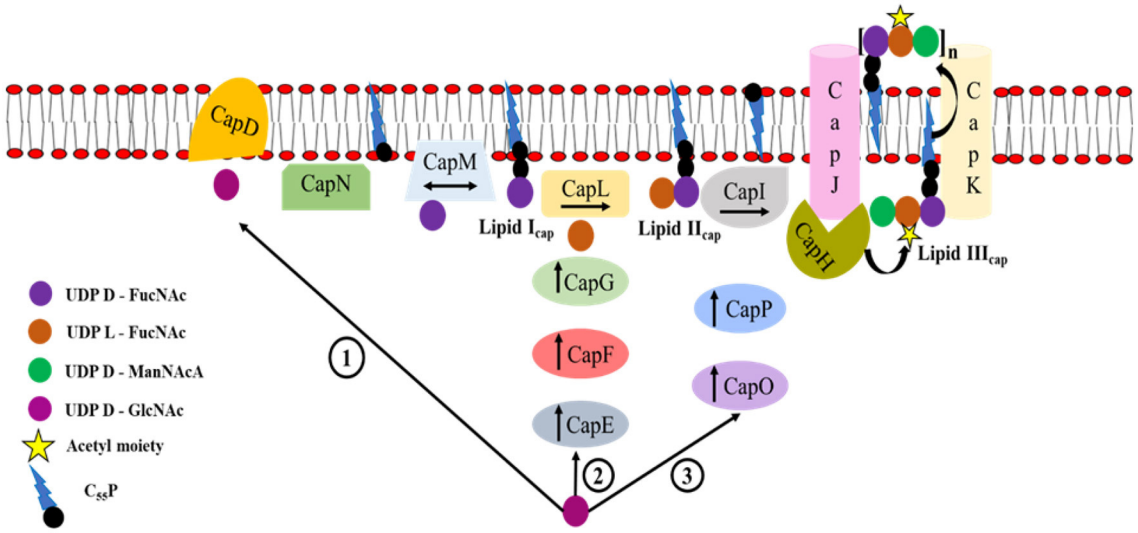

FIGURE 6
Cascade of capsule biosynthesis. The pathway for capsule (CP) biosynthesis occurs in the cytoplasm *via* three distinct reactions in which the universal cell envelope substrate UDP-D-N-acetylglucosamine (UDP-D-GlcNAc) is converted into the three different nucleotide-coupled sugars: (1) UDP-N-acetyl- D-fucosamine (UDP-D-FucNAc) by the enzymes CapD and CapN, (2) UDP-N-acetyl-L-fucosamine (UDP-L-FucNAc) by the enzymes CapE, CapF, and CapG, and (3) UDP-N-acetyl-D-mannosaminuronic acid (UDP-D-ManNAcA) by the enzymes CapP and CapO. The enzymes CapK and CapJ translocate the modified precursor to the outer surface of the cell membrane where polymerization occurs (modified from Rausch et al., 2019).

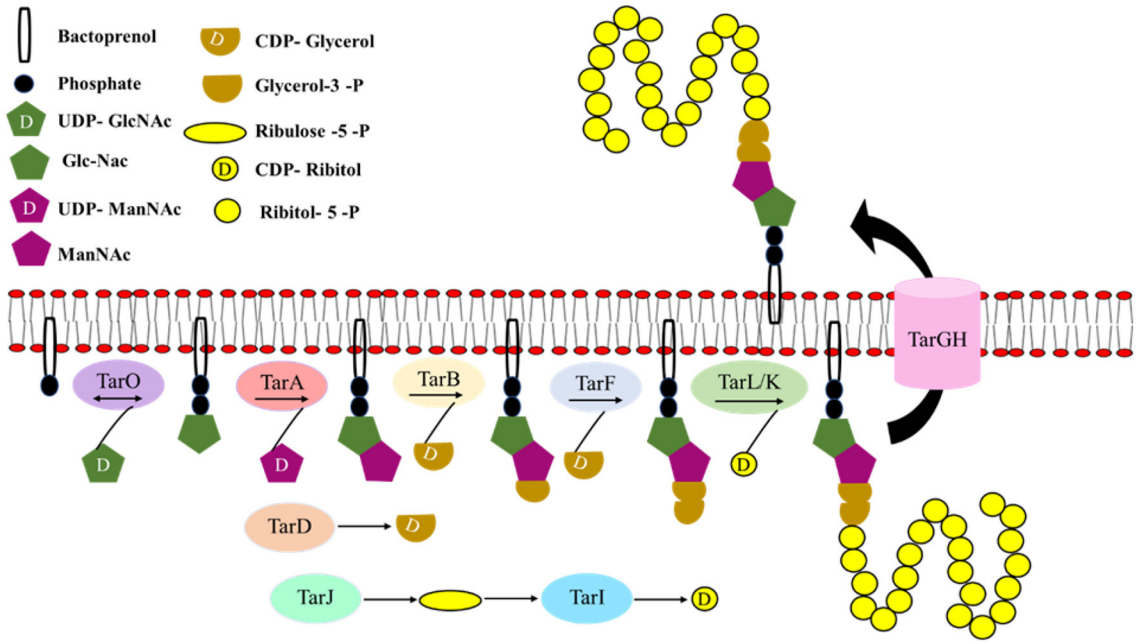

FIGURE 7
Cascade of wall teichoic acid biosynthesis. The first three steps of the WTA (wall teichoic acid) biosynthetic pathway are catalyzed by the enzymes TagO (TarO), TagA (TarA), and TagB (TarB), respectively leading to the formation of the linkage unit which comprises of a single phosphoglycerol unit from CDP-glycerol to the C4 hydroxyl of ManNAc-β1,4-GlcNAc disaccharide. TarF adds one additional glycerol phosphate unit to the linkage unit. The assembly of the poly(ribitol) phosphate main chain is carried out by one of the two bifunctional poly(ribitol) phosphate primases/polymerases known as TarK/TarL. Once the polymerization is completed in the cytoplasm, glycosylation occurs and the polymer is flipped out by an ABC-dependent transporter complex TarGH followed by which ligation to the cell wall occurs (modified from Swoboda et al., 2010).

together synthesize the CDP-ribitol substrate that is utilized by TarL. TarL attaches more than 40 ribitol phosphates to complete the polymer synthesis (Brown et al., 2013). *tarK* gene is highly homologous to the *tarL* gene, suggesting that it might have the same enzymatic function. The reason for the presence of two homologous sets of *tarIJL* genes (*tarI'J'K* genes) with the same

function (Qian et al., 2006) in *S. aureus* is still not clear; however, Meredith et al. (2008) have reported that *tarK* can nullify the loss of *tarL* and have an enzymatic function similar to *tarL* the gene product. However, strains that produced only TarL or TarK produced two electrophoretically distinct poly (ribitol) phosphate WTAs, known as L-WTA or K-WTA, respectively. K-WTA was found to be significantly shorter than L-WTA based on PAGE analysis. *tarK* gene expression is negatively regulated by *agr* quorum sensing system. As the expression of *tarK* can shorten K-WTA length by 50%, it is presumed that WTA chain length is dynamically regulated by *S. aureus* between pro-adhesion and low adhesion state to promote adhesion and dispersal of biofilm during different stages of infection. Once the polymerization is completed in the cytoplasm, glycosylation occurs and the polymer is flipped out by an ABC-dependent transporter complex TarGH followed by which ligation to the cell wall occurs (Swoboda et al., 2010).

4.1.4.1. Regulation of WTA biosynthesis

Highly pathogenic CA-MRSA strains express the increased level of WTAs by upregulating the TarH ATPase subunit of the TarGH ABC transporter. This "WTA-high phenotype" is expressed in highly virulent strains in which the *agr* quorum sensing system is upregulated (van Dalen et al., 2020). Wanner et al. (2017) have reported increased skin abscess formation, T-cell proliferation, and IFN-γ production in mouse skin models infected with WTA-high phenotype strains. When *agr* is active, L-WTA is expressed resulting in long chains that may aid in colonization and infection (Meredith et al., 2008). Cross-regulation of WTA and CP biosynthesis is another important factor as both processes compete for C_{55}P lipid carriers. CP production is thus very tightly regulated and only expressed in post exponential phase that too in a fraction of the population in order to ensure sufficient C_{55}P lipid carrier for WTA production which is an inevitable component of the bacterial cell wall (Keinhoerster et al., 2019).

4.2. Regulation of virulence factor production

4.2.1. agr locus

The *agr* locus (first reported by Peng et al., 1988) is 3.5 kb in size and consists of two divergent promoters P2 and P3 that generates the transcripts of RNAII and RNAIII, respectively. RNAII locus comprises of four genes namely, *agrB*, *agrD*, *agrC*, and *agrA*. *agrD* transcript encodes a 7–9 amino acid long autoinducing peptide (AIP), which also plays a role in extracellular quorum sensing signal in *S. aureus* (Ji et al., 1995). AgrB is a multifunctional endopeptidase that is responsible for maturation (thiolactone modification and C terminal cleavage) and the export of AIP across the cell membrane. AgrC and AgrA constitute the two-component signal transduction system, of which AgrC is a membrane-bound histidine kinase, which is auto-phosphorylated upon the binding of AIP. It then trans-phosphorylates the response regulator AgrA. Activated AgrA can bind to the P2 and P3 promoters of *agr* locus and can regulate the expression of downstream genes (Novick et al., 1995; Queck et al., 2008; Le and Otto, 2015; Figure 8).

The majority of the virulence factors that are under the control of *agr* system are regulated by RNAIII. It is a messenger RNA that contains the *hld* gene for delta toxin or delta hemolysin (Janzon et al., 1989). It also activates the transcription of the *hla* gene for an alpha toxin or alpha hemolysin. RNAIII controls the expression of surface proteins, such as Protein A, coagulase, and fibrinogen binding protein, and repressor of toxin (Rot) protein by antisense base pairing with 5′ untranslated regions (5′ UTR) and forming RNA duplexes (Boisset et al., 2007). Rot protein binds to the promoter of many exoproteins (α, β, and γ hemolysin), enterotoxins (Toxic shock syndrome toxin), and leukocidins, inhibiting their transcription. The inhibition of Rot and surface proteins by RNA III leads to an upregulation of virulence factors along with the dispersal of biofilm. AgrA can also upregulate the transcription of phenol-soluble modulins *psmα* and *psmβ* operons in an RNAIII-independent manner (Queck et al., 2008; Le and Otto, 2015).

agr system is a global regulatory system of *S. aureus* for the upregulation of virulence factors, which aids *S. aureus* to cause several types of infections (Li S. et al., 2014; Tuchscherr and Löffler, 2016) and downregulation of surface proteins for disruption of biofilm. During the initial course of bacterial infection when the cell density is low, *agr* system is downregulated, resulting in an increased production of adhesins and surface proteins. Once colonization is established and nutrients become limited, the upregulation of degradative exoenzymes and toxins mediated by *agr* occurs, which helps not only to acquire nutrition for the cell but also to evade the host immune system (Fowler et al., 2004; Cheung et al., 2014). Yarwood and Schlievert (2003) have reported thicker and smoother biofilms on medical devices formed by *agr* mutant strains, leading to chronic and persisting infections in the host due to their inability to disseminate from the patient's body (Shopsin et al., 2010). The ability of *Staphylococcus aureus* to form biofilm in chronic relapsing infections associated with medical devices such as urinary catheters, intravenous catheters, and orthopedic prosthetics (Singh and Ray, 2014) shields the bacteria from the host immune system as well as antibiotics (Waters et al., 2016).

S. aureus strains can be classified into four agr groups (agrI, agrII, agrIII, and agrIV) based on the *agrD* gene (which encodes the synthesis of AIPs) and *agrC* gene (which encodes the receptor of *agr* TCS). The four AIP molecules are similar enough to bind with the AgrC receptor of the different groups but cannot activate the AgrA protein unless it is activated by the AIP of a similar group (Jabbari et al., 2012). Ikonomidis et al. (2009) and Khoramrooz et al. (2016) have reported that strains of *agr* group II and *agr* group III are more potent biofilm producers. Nichol et al. (2011) have also established a relationship between antibiotic resistance and *agr* groups in *S. aureus*. *agr* group I is widely associated with CA-MRSA genotypes, whereas *agr* group II is associated with HA-MRSA genotypes in humans.

4.2.1.1. Regulation of *agr* locus

(i) **SarA family**

It is encoded by the *sar* gene present in the *sar* locus (an important global virulence regulon that plays a major role in growth, biofilm formation, and production of toxins in *S. aureus*). It binds to P2–P3 intragenic region and activates *agr* transcription while another protein Sar R (which also binds to P2-P3 intragenic region) represses the transcription of *agr* (Reyes et al., 2011). *sar* locus possesses ∼50,000 copies of the *sarA* gene per cell and it can

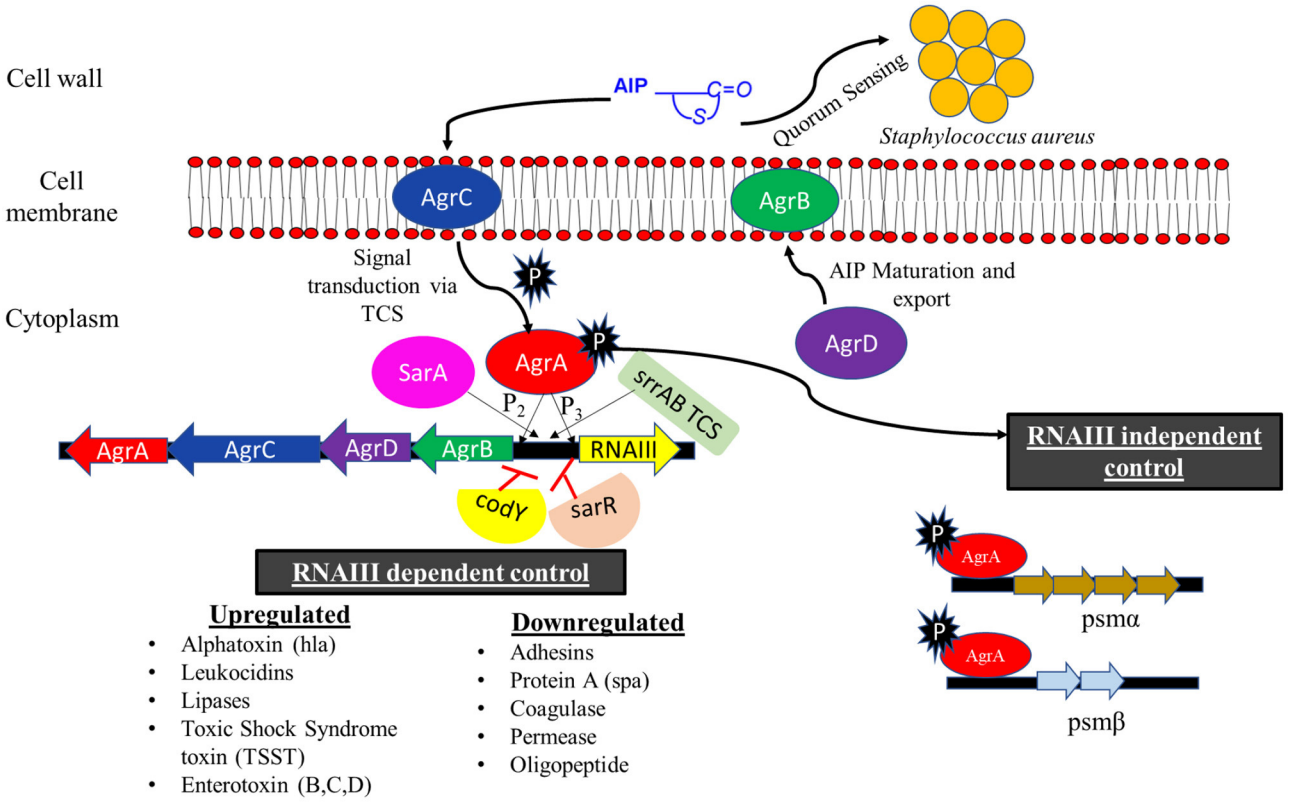

FIGURE 8
Regulation of *agr* (accessory gene regulator) two-component system. Autoinducing peptide (AIP) synthesized by AgrD is transported across the cell membrane by transmembrane protein AgrB. AIP acts as a signal for quorum sensing in *S. aureus* and can also activate the AgrAC TCS. The activated AgrA can bind to the P2 and P3 promoter of *agr* locus and initiate the transcription of *agrACDB* genes and RNAIII transcript, respectively. RNAIII transcript can mediate the expression of various virulence and biofilm disruption genes. The activated AgrA initiates the transcription of *psmα* and *psmβ* in RNAIII independent manner. SarA and SrrAB positively regulate the *agr* locus whereas codY and sarR exhibit a negative effect (modified from Le and Otto, 2015; Tan et al., 2018).

regulate the expression of nearly 120 genes (encoding virulence, cell wall associated, and extracellular proteins) of *S. aureus* (Cheung et al., 2008; Fujimoto et al., 2009). It consists of three transcripts i.e., *sarA, sarB,* and *sarC* under the control of P1, P2, and P3 promoters, respectively (Cheung and Manna, 2005). SarA is a DNA binding protein that can bind to the P2 promoter of *agr* locus and upregulate the transcription of RNA III. Hence, the *sar* locus can directly activate the *agr*-mediated quorum sensing in *S. aureus via* the P2 promoter of *agr* locus (Rechtin et al., 1999; Sterba et al., 2003; Roberts et al., 2006). α toxin, phenol-soluble modulins, and Panton Valentine Leukocidin (PVL), which are responsible for causing critical infection in CA-MRSA, are regulated by SarA (Bronner et al., 2000; Dumitrescu et al., 2011; Zielinska et al., 2011). Production of extracellular proteases such as serine protease, cysteine protease, metalloprotease, and staphopain are also negatively regulated by SarA (Karlsson and Arvidson, 2002). It can bind to various promoters *viz*., *sarA*, *mecA*, and *sarR* under the same conditions and at the same time point as revealed by DNA affinity capture assay (DACA; Kim et al., 2022). *Sar* locus has been reported to play a major role in regulating antibiotic resistance mechanisms. Proteomic analysis revealed that *mecA* expression was reduced in *sarA* deficient mutant, the exact reason for which is still unknown. However, this might be a reason for antibiotic susceptibility in *sarA* deficient mutant strains as both genes are involved in biofilm formation and PBP2a expression (Kim et al., 2022). Also, the inactivation of *sarA* has led to a reduction in ciprofloxacin and vancomycin resistance (Lamichhane-Khadka et al., 2009). Sequence analysis of clinical strains of various lineage revealed that the *sarA* gene was highly conserved unlike the other global virulence regulator AgrA which had various mutations (Kim et al., 2022). SarA regulates the post-transcriptional expression of *spa* and collagen adhesion genes during the exponential growth phase by binding to the target mRNA. It changes the turnover as well as accounts for the stability of their transcripts (Roberts et al., 2006; Morrison et al., 2012). Unlike *agr* locus, whose transcription is initiated majorly in the exponential phase, SarA protein levels are constant throughout the growth phases of the bacterium (Cheung and Manna, 2005; Arya and Princy, 2013).

S. aureus has various SarA paralogs i.e., SarR, SarS, SarT, Rot, SarU, SarV, SarX, MgrA, and MarR, which are either inhibitor or stimulator of each other, and indirectly contributes to virulence, biofilm production, autolysis, antibiotic resistance, and metabolic processes (Trotonda et al., 2008; Ballal and Manna, 2009). Northern blot and transcriptional fusion analysis have confirmed that SarV, a 116-residue long polypeptide, which is a homolog of SarA and regulator of cell lysis, is regulated by *sarA* as well as *mgrA*. Various virulence and autolysis genes have been reported to be under the regulation of *sarV* (Cheung et al., 2008; Trotonda et al., 2009).

SarR, a 13.6 kDa dimeric protein, is a repressor of SarA, which regulates *agr* expression directly by binding to the intragenic region of the P2–P3 promoters, where SarA also binds but with less affinity than SarR. SarR binds to *sarA* or the promoters of target genes, repressing the expression of *sarA*, *agr*, *hla*, *hlb*, and *spa* during the post-exponential growth phase (Cheung and Zhang, 2001; Manna and Cheung, 2001; Oscarsson et al., 2005; Arya and Princy, 2013).

SarS, a 250-residue-long polypeptide with 64% homology with SarA, is a repressor of *hla* transcription and activator of protein A (*spa*; Manna and Ray, 2007). Rot, a 166 amino acid long residue, is an additional regulatory protein that modulates the expression of *sarS*, a transcriptional regulator of virulence genes. Rot protein has been reported to negatively regulate the production of various toxins such as lipases, serine proteases, α toxin, β toxin, cysteine proteases, and several other proteases, suggesting that *rot* acts downstream of the *agr* locus and indirectly upregulates cell wall synthesis. Activation of *agr* results in the production of RNA III which inhibits the production of *rot* (Said-Salim et al., 2003; Schmidt et al., 2003). Treatment with protease inhibitors such as cysteine protease inhibitor E-64 or staphostatin SspC, a specific inhibitor of staphopain B, is necessary to restore biofilm formation in a *rot* mutant (Mootz et al., 2015). SarA and Rot both repress protease production and are thus important regulators of biofilm formation.

SarT, another 118-residue-long homologous protein of SarA that downregulates *hla* and RNA III expression, is negatively regulated by *sarA* (Manna and Cheung, 2003). SarU, a 247- residue-long protein with a molecular mass of 2.92 kDa, is adjoining to *sarT* but is transcribed in the opposite direction. Mutational analysis revealed an elevated expression of *sarU* in *sarT* mutants indicating that *sarU* is negatively regulated by *sarT*. *sarU* mutants exhibited a lower RNAII and RNAIII expression as compared to the parental strain as proved by transcriptional and northern blot analysis. This observation suggests that *sarU* indirectly activates *agr* locus by upregulating RNAIII expression and altering the expression of *agr*-mediated virulence genes (Liu et al., 2006; Arya and Princy, 2013). Finally, the global regulator MgrA (member of the SarA protein family) acts as a repressor of eight cell wall-anchored proteins. *mgrA* mutants exhibited an increased biofilm formation with a loss of bacterial clumping (Schilcher and Horswill, 2020).

(ii) **SrrAB**

SrrAB is a two-component system where SrrA is a 28 kDa, 241 amino acid long response regulator, and SrrB is a 66 kDa, 583 amino acid long histidine kinase. Low oxygen levels and redox environmental conditions such as pH serve as a signal for SrrAB TCS. Upon receiving the signal, the membrane-bound SrrB auto-phosphorylates at a conserved histidine residue. This phosphate group is then transferred to the aspartate residue of cytoplasmic SrrA. SrrA has been reported to bind to both P2 and P3 promoters of *agr* system, positively affecting its activity (Pragman et al., 2004; Tan et al., 2018).

(iii) **CodY**

The nutritional status of the cell greatly impacts biofilm formation and virulence factors production, which is regulated *via* CodY (global transcriptional regulator) in *S. aureus* (Schilcher and Horswill, 2020). CodY has been reported to strongly repress agr locus (Majerczyk et al., 2008) not by binding to the P2 and P3 promoter regions but rather with a region within the *agrC* gene (Majerczyk et al., 2010). However, this had no effect on *agrA* expression. On the contrary, Roux et al. (2014) reported *in vitro* binding of CodY to the P2 and P3 promoter of *agr* locus but with low affinity. Recently, CodY exhibited repression of *rsaD*, a small regulatory RNA (sRNA) responsible for causing cell death regulation during weak acid stress has been reported, which eventually causes eDNA release and biofilm formation (Augagneur et al., 2020).

4.2.2. sae locus

The *sae* (staphylococcal accessory element) system was first identified by Giraudo et al. (1994) while studying a Tn551 insertional mutant in which the production of exoproteins such as nuclease, coagulase, α hemolysin, and β hemolysin was found to be altered. The *sae* operon consists of four genes, i.e., *saeP*, *saeQ*, *saeR*, and *saeS*, which are under the regulation of P1 and P3 promoters. The *sae* locus primarily comprises of a two-component system in which SaeR is the response regulator and SaeS is the histidine kinase (Giraudo et al., 1999). This system is activated by external stimuli such as H_2O_2 and α defensins and repressed under low pH and high NaCl concentrations, both resulting in membrane perturbation (Geiger et al., 2012; Haag and Bagnoli, 2015). The signal is sensed by SaeP which interacts with the extracellular linker peptide of SaeS leading to its autophosphorylation at His131 which then transphosphorylates SaeR at Asp51. SaeR can now bind to SBS (SaeR binding sequence), leading to the upregulation of *saeR*-mediated virulence genes (Figure 9).

Very little was known about the role of auxiliary proteins SaeP and SaeQ in Sae TCS activity and disease progression. Collins et al. (2020) generated mutants of *saeP*, *saeQ*, and *saeP-saeQ* double mutant of the USA300 strain of *S. aureus* to analyze their function. The survival of *S. aureus* USA300Δ*saeP* increased compared to the wild type USA300 strain post phagocytosis by neutrophils probably due to increased expression of bicomponent leukocidins by the former. The USA300Δ*saeQ* also yielded a similar phenotype but the neutrophil interaction results were comparatively less significant. However, the *saeP-saeQ* double mutant strain (USA300Δ*saePsaeQ*) exhibited a drastic increase in neutrophil survival and virulence during murine bacteremia as compared to the USA300Δ*saeP* single mutant strain. These observations suggested the role of SaeP in the survival of bacterium after phagocytosis by neutrophils and the combined effect of SaeP and SaeQ in *in vivo* pathogenesis.

Flack et al. (2014) have reported that SaeS more specifically detects α-defensin 1/Human neutrophil peptide 1 (HNP-1) and human polymorphonuclear leucocytes (PMNs). SaeR and SaeS are transcribed *via* the P3 promoter located within the *saeQ* coding sequence. The P1 promoter is located upstream of the *saeP* gene and can transcribe all four genes of the locus. The P1 promoter has been reported to exhibit 2–30 times higher activity compared to the P3 promoter. Jeong et al. (2011) have reported that only basal level expression of *saeRS* genes from the P3 promoter is sufficient

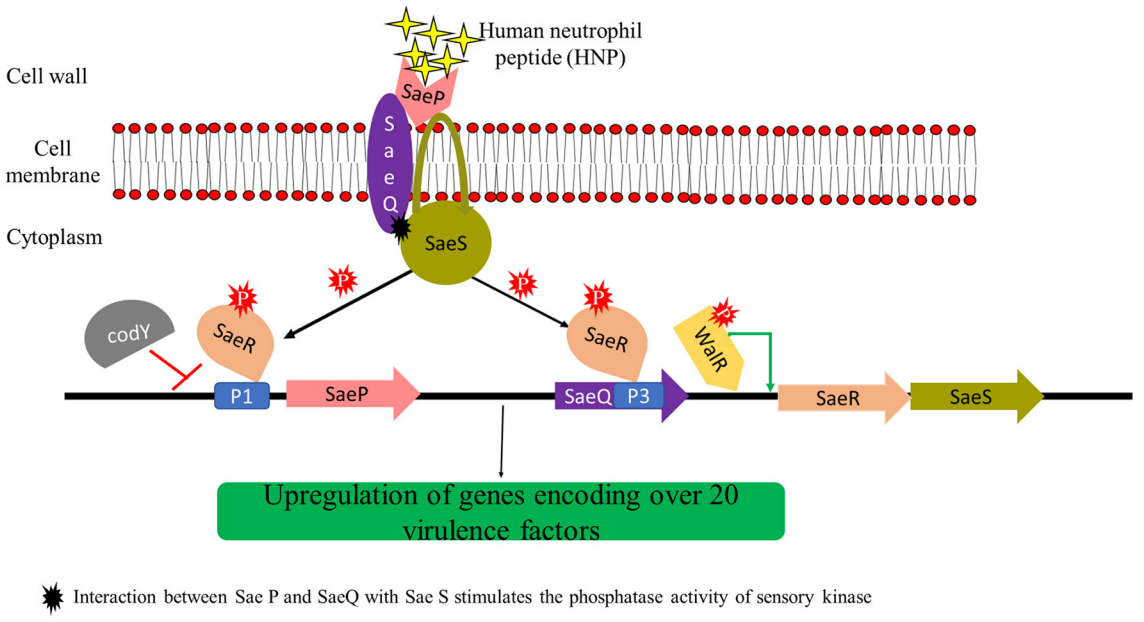

FIGURE 9
Regulation of *sae* (Staphylococcal accessory element) two-component system. Inducing molecules such as Human Neutrophil peptide 1 (HNP-1) are sensed by the extracellular membrane protein SaeP which interacts with the extracellular linker peptide of the cytoplasmic domain SaeS, activating the SaeRS TCS. Activated SaeR can induce the transcription of *saePQRS* (and genes encoding over 20 virulence factors) *via* P1 and P3 promoters. SaeP and SaeQ can form a protein complex with SaeS and induces the sensory kinase phosphatase activity resulting into the dephosphorylation of activated SaeR leading to the downregulation of virulence genes which are regulated by *saeR*. All these interactions are based on predictions (modified from Liu et al., 2016).

for activation of *sae* target genes and exoprotein production. P1 promoter has two *saeR* binding sites and thus it can be auto-induced by *saeR* transcripts produced by basal level expression of *saeRS* TCS *via* P3 promoter (Figure 7). The target genes of the *sae* system have been bifurcated into two classes: Class I target genes require high levels of phosphorylated SaeR for its activation, *viz.*, *fnbA*, *coa*, P1 promoter, *sae*, and *eap*, while Class II target genes require basal/lower levels of phosphorylated SaeR for its activation, *viz.*, *hla* and *hlb* genes (Mainiero et al., 2010; Haag and Bagnoli, 2015). SaeP and SaeQ have been reported to form a protein complex with SaeS and induce the sensory kinase phosphatase activity resulting in dephosphorylation of activated SaeR which downregulates the virulence genes regulated by *saeR* (Jeong et al., 2012; Haag and Bagnoli, 2015).

Depending on growth conditions, *sae* system can both positively (upregulation of biofilm forming genes, i.e., *hla*, *hlb*, *coa*, *emp*, *eap*, *fnBPA*, and *fnBPB*) and negatively (upregulation of biofilm dispersal factors such as nucleases and proteases) affect biofilm formation (Caiazza and O'Toole, 2003; Johnson et al., 2008; O'Neill et al., 2008; Huseby et al., 2010; McCourt et al., 2014; Zapotoczna et al., 2015; Liu et al., 2016). Moormeier et al. (2014) have reported that *nuc* transcription is positively regulated by the SaeRS system, as both *sae* and *nuc* mutant were unable to cause the dispersal of biofilms, which likely explains the degradation of eDNA during the dispersal stage of biofilm formation. Moreover, SaeS has been reported to exhibit polymorphism across *S. aureus* strains as a point mutation can lead to hyperactivation of SaeRS TCS leading to the inability of such strains to form robust biofilms (Mainiero et al., 2010; Cue et al., 2015). *saeRS* locus also acts synergistically with *sarA* to inhibit the production of extracellular proteases, resulting in an improved ability for biofilm formation (Arya and Princy, 2013).

SaeRS system positively regulates NETs (Berends et al., 2010; McDonald et al., 2012), SCIN, CHIPS (Rooijakkers et al., 2006), leukocidins (Münzenmayer et al., 2016), α hemolysin (Hla; Nygaard et al., 2013), pro-inflammatory cytokines, proteases, and toxins production (Watkins et al., 2011; Zurek et al., 2014; Cho et al., 2015). However, *S. epidermis,* which also possesses a SaeRS two-component system and is closely related to *S. aureus,* does not possess these virulence genes (Handke et al., 2008; Ravcheev et al., 2011). This indicates that *S. aureus* has acquired these virulence genes during evolution, which are being maintained by it under the control of the SaeRS two-component system (Liu et al., 2016).

4.2.2.1. Regulation of *sae* system

Transcription of *sae* operon has been reported to be modulated by other global and local regulatory systems, which are mentioned as follows:

(i) **agr system**

The majority of toxins and exoproteins under the control of *agr* and *sae* operons are similar; however, there are several reports which suggest that *agr* and *sae* are independent of each other (Liu et al., 2016). *sae* operon does not possess AgrA or RNAIII binding sites, indicating that activation of *agr* operon should not have any significant effect on *sae* operon. Some of the virulence genes under the regulation of *agr* and *sae* are transcribed in opposite manner. *coa* and *fnbA* genes are repressed, whereas the *cap* gene

is activated by *agr* operon. On the other hand, *coa* and *fnbA* genes are activated and the *cap* gene is repressed by the *sae* operon (Dassy et al., 1993; Wolz et al., 1996; Saravia-Otten et al., 1997; Luong et al., 2002; Steinhuber et al., 2003). Toxic shock syndrome toxin (TSST) is positively regulated by both *agr* and *sae* operon. However, *agr* mutants did not show impairment of TSST toxin production as observed in *sae* mutants (Baroja et al., 2016). Despite the positive regulation of *hla* expression *via* RNAIII activation by *agr* locus *in vitro*, *sae* operon has been reported to be inevitable for *in vivo* toxin production (Novick et al., 1993; Goerke et al., 2001).

(ii) **σ_B**

In the case of *sae* operon, σ_B has been reported to cause downregulation of *saePQRS* and *sae* target genes (*hla*, *nuc*, *splABCDEF*, and *hlgABC*; Mitchell et al., 2013) mostly *via* regulatory proteins or small non-coding RNAs which may be positively regulated by σ_B (Bischoff et al., 2004). No evidence of cross-talk between *sae* locus and σ_B has been reported, suggesting the fact that both are independent regulatory systems (Goerke et al., 2005; Liu et al., 2016).

(iii) **CodY**

Branched-chain amino acids (BCAA) *viz*., isoleucine, leucine, valine, and GTP are the key metabolites that activate CodY as a DNA-binding protein and bind to the sequence motif (AATTTTCWGAAAATT) of chromosomal DNA. CodY and SaeR can both bind to the *sae* P1 promoter in an opposite manner where one is the repressor and the other is the activator, respectively. CodY and SaeR have been reported to compete for binding to the *saeP* regulatory region. CodY is a stronger repressor of *sae* P1 promoter as compared to Rot. When nutrients are abundant, CodY inhibits the transcription of *saeRS* operon and *sae* dependent virulence genes but with the depletion of nutrients, the affinity of CodY to *sae* P1 promoter is lost resulting in an upregulation of *sae* operon. The subsequent increase in the production of cytotoxic factors helps *S. aureus* to combat the host immune system by destroying neutrophils. Pendleton et al. (2022) have reported that the cell membranes of *codY* mutant strains of *S. aureus* have a higher percentage of branched-chain fatty acids (BCFAs) as compared to the cell membranes of wild-type strains. This observation suggests the possibility of post-transcriptional regulation of the Sae system by the global repressor codY. Disruption of the *lpdA* gene which encodes dihydrolipoyl dehydrogenase (an important enzyme in BCFA synthesis) results in a reduction of SaeS kinase activity as well as the response regulator SaeR-P. This ultimately leads to a reduction in exotoxin secretion and attenuation of virulence. Thus, CodY acts as a nutritional checkpoint protein which ensures that the *saePQRS* operon is activated only when cytolytic and pore-forming toxins are to be secreted to acquire nutrition from the host and functions as a switch between commensal and invasive lifestyles of *S. aureus* (Mlynek et al., 2018).

(iv) **WalRK**

WalRK is one of the major two-component system of *S. aureus* which plays a role in cell wall metabolism and cell viability (Dubrac and Msadek, 2004; Dubrac et al., 2007). When the response regulator, WalR is produced in large amounts in its active form then the upregulation of *sae* operon has been observed. However, this upregulation was terminated when SaeRS two component system was deleted (Delauné et al., 2012). These results indicated that WalRK positively affects SaeRS TCS; however, the exact mechanism by which it occurs is still unknown (Liu et al., 2016).

(v) **Fur regulon**

Fur regulon of *S. aureus* is responsible for iron uptake, biofilm formation, and anti-oxidative stress response (Hantke, 1981; Litwin and Calderwood, 1993; Horsburgh et al., 2001; Johnson et al., 2005; Richardson et al., 2006; Lee and Helmann, 2007). *saeRS* transcription from both P1 and P3 promoters is downregulated in *fur* mutant as well as when iron from an exogenous source is supplied in media (*fur* is downregulated as iron is freely available), suggesting that Fur may be a positive regulator of *saeRS* (Johnson et al., 2011). However, the direct effect of Fur regulon on *saeRS* transcription is still unclear because *sae* operon does not possess Fur binding sites (Cho et al., 2015; Liu et al., 2016).

(vi) **Rot**

Rot, a member of the SarA protein family, is known to repress the expression of toxins (such as *hla*) in *S. aureus* (McNamara et al., 2000; Li and Cheung, 2008). Li and Cheung (2008) have reported that Rot can bind to the P1 promoter of the *sae* locus and repress it suggesting that Rot represses *hla* transcription *via* the P1 promoter of the *sae* locus. However, as the P1 promoter is not involved in the transcription of *sae* target genes (Jeong et al., 2011), the observations of Li and Cheung (2008) seem to be unlikely (Liu et al., 2016).

(vii) **Fak system**

Fatty acid kinase (*fakA* and *fakB1/fakB2*) mutants of *S. aureus* have been reported to exhibit a decrease in α hemolysin production as well as other *sae* target genes, indicating positive regulation of SaeRS TCS by the Fak system. It is believed that the acyl-PO_4 group of FakB may donate the phosphoryl group to SaeR leading to its activation (Parsons et al., 2014).

(viii) **ScrA**

A novel protein ScrA acts *via* the SaeRS TCS to regulate virulence gene expression in *S. aureus*. ScrA protein acts as an intermediate between ArlRS and SaeRS systems (Wittekind et al., 2022). ArlRS TCS regulates genes involved in adhesion (*ebh* and *sdrD*), virulence (*nuc*, *lukA*, and *esxA*), and transcriptional factors (*sarV* and *mgrA*; Crosby et al., 2020). *scrA* expression is increased directly *via* the ArlR response regulator or indirectly *via* other regulators such as *mgrA*. ScrA has been hypothesized to stimulate SaeS kinase activity. This activates the SaeRS TCS which upregulates the production of adhesins and hemolysins and downregulates proteases which in turn increases cellular aggregation, biofilm formation, and hemolysis. The activated SaeRS TCS acts as a feedback inhibitor and directly or indirectly represses *scrA* (Wittekind et al., 2022).

5. Conclusion

S. aureus being a normal microflora of humans will always coexist with mankind. The extensive use of antibiotics across the world has led to the emergence of more resistant strains such as MRSA requiring novel antibiotics and treatment strategies. The ease of infection, high mortality rates, lack of suitable animal models, and increase in antibiotic resistance in MRSA have proved to be major hurdles to advances in clinical research. *S. aureus* pathogenesis is more complex as it is not dependent on a single major virulence factor that leads to disease progression. Secretion of a diverse array of virulence factors during its course of infection poses a major challenge in both drug and vaccine development. Though humans have made outstanding achievements in understanding the pathogenesis of MRSA, there are still gaps in knowledge and some important challenges to overcome. First, the adaptive immune evasion mechanisms post *S. aureus* infections remain unknown. Second, a vaccine targeting multiple factors against *S. aureus* is yet not successfully developed. Apart from these, a better understanding of bacterial pathogenesis, prevention of transmission and infection, and advancement in diagnosis requires focused concentration by researchers, policymakers, funding agencies, and well-coordinated multidisciplinary approaches that may help control the transmission of this highly successful pathogen.

Author contributions

HP has written the article. SR has designed the concept and edited the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Awareness and knowledge of female genital schistosomiasis in a population with high endemicity: a cross-sectional study in Madagascar

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Introduction: Female genital schistosomiasis (FGS) is a neglected disease with long-term physical and psychosocial consequences, affecting approximately 50 million women worldwide and generally representing an unmet medical need on a global scale. FGS is the chronic manifestation of a persistent infection with *Schistosoma haematobium*. FGS services are not routinely offered in endemic settings with a small percentage of women at risk receiving adequate care. Madagascar has over 60% prevalence of FGS and no guidelines for the management of the disease. This study aimed to determine FGS knowledge among women and health care workers (HCWs) in a highly endemic area of Madagascar.

Methods: A convenience sampling strategy was used for this cross-sectional study. Descriptive statistics including proportions and 95% confidence intervals (CI) were calculated, reporting socio-demographic characteristics of the population. Knowledge sources were evaluated descriptively. Binary Poisson regression with robust standard errors was performed; crude (CPR) and adjusted prevalence ratio (APR) with 95% CIs were calculated.

Results: A total of 783 participants were included in the study. Among women, 11.3% ($n = 78$) were aware of FGS while among the HCWs 53.8% ($n = 50$) were aware of FGS. The highest level of knowledge was observed among women in an urban setting [24%, ($n = 31$)] and among those with a university education/vocational training [23% ($n = 13$)]. A lower APR of FGS knowledge was observed in peri-urban [APR 0.25 (95% CI: 0.15; 0.45)] and rural [APR 0.37 (95% CI 0.22; 0.63)] settings in comparison to the urban setting. Most HCWs reported other HCWs [40% ($n = 20$)] while women mainly reported their family [32% ($n = 25$)] as being their main source of information in the 6 months prior to the survey.

Discussion and conclusions: Our study shows limited awareness and knowledge of FGS among population groups in the highly endemic Boeny region of

Madagascar. With this study we contribute to identifying an important health gap in Madagascar, which relates to a disease that can silently affect millions of women worldwide. In alignment with the targets of the NTD roadmap, addressing schistosomiasis requires a paradigm shift for its control and management including a greater focus on chronic forms of the disease.

KEYWORDS

female genital schistosomiasis, women's health, public health, unmet medical needs, awareness, knowledge

Introduction

Awareness and knowledge of diseases are key elements for their prevention, early detection and successful management (Aerts et al., 2020). Neglected tropical diseases (NTDs) are a group of diseases suffering, more than others, from lack of awareness and knowledge among both health policy makers as well as health care users and providers (Ortu and Williams, 2017).

Lack of awareness and knowledge about these diseases among stakeholders can contribute to the emergence of unmet medical needs (UMN) (Prusty, 2014; Merki-Feld et al., 2018; Vreman et al., 2019). UMN can be generally defined as conditions without satisfactory diagnostics, prevention or treatment (Vreman et al., 2019). While UMN are a highly debated public health issue (Zhang et al., 2021), it is well recognized that they ultimately contribute to the increasing burden of diseases (Chen et al., 2022), particularly concerning those that are rare (As-Sanie et al., 2019) or less prominent in the global medical sphere, such as NTDs (Chami and Bundy, 2019). Yet, these conditions could potentially be effectively managed with minimal investments.

Human schistosomiasis is a vector-borne NTD with a zoonotic life cycle that occurs primarily in tropical areas. Schistosomiasis prevalence is particularly high in sub-Saharan Africa (SSA) with more than 80% of the global disease burden (Boissier et al., 2016; McManus et al., 2018). Schistosomiasis is caused by six different species of the trematode schistosome, of which *Schistosoma mansoni* and *Schistosoma haematobium* are the most frequent worldwide (Gryseels et al., 2006). The disease leads to chronic inflammations induced by the deposition of *Schistosoma* eggs that can calcify and lead to major detrimental health outcomes (McManus et al., 2018).

The NTD roadmap, released by the World Health Organization (WHO) in 2021 (World Health Organization, 2021), targets the elimination of the disease as a public health problem by 2030 in all endemic countries. Progress on control has been made through vertical programs based on mass drug administration (MDA) with praziquantel, used as preventive chemoprophylaxis in school aged children. These strategies systematically neglected adults, favouring the conditions for the development of chronic forms of the disease especially in highly endemic contexts (Kura et al., 2020; Gruninger et al., 2023). Chronic intestinal schistosomiasis caused by *S. mansoni* can lead to hepato-splenomegaly and portal hypertension while chronic urogenital schistosomiasis caused by *S. haematobium* increases the risk of developing squamous bladder cancer and can lead to male and female genital schistosomiasis.

Female genital schistosomiasis (FGS) is the chronic manifestation of schistosomiasis induced by a persistent infection with *S. haematobium* through the deposition and calcification of eggs in the female genital tract (Mazigo et al., 2022a). The global burden of FGS is unknown most likely due to under-reporting (Engels et al., 2020), but it is estimated that it affects 40 to 56 million women and girls worldwide (Hotez et al., 2019a). On the basis of current available data, between 33% and 75% of women and girls suffer from infection with *S. haematobium* and are at risk of developing FGS (Hotez and Whitham, 2014). Furthermore, FGS has been shown to increase the risk of HIV infection, and is suspected to play a role in the onset and/or progression of cervical cancer (Hotez et al., 2019a,b; Patel et al., 2021).

FGS induces cervical lesions and can lead to ectopic pregnancies and infertility (Kjetland et al., 2012). Moreover, it has been linked to psychosocial consequences, such as stigma and depression as well as a loss of work productivity and therefore reduced income (Hotez and Whitham, 2014). The latter contributes to the perpetuation of the vicious cycle of poverty frequently associated with NTDs (de Rijk et al., 2021). FGS shares common clinical features with sexually transmitted diseases (STDs) such as pain, itching or vaginal discharge (Mazigo et al., 2021) frequently leading to misdiagnosis, inappropriate treatment, social stigma, and further risk of under detection and underreporting. Limited awareness and treatment of FGS has been described both among HCWs and the general population in several endemic countries such as Ghana, Tanzania, and Cameroon (Kukula et al., 2019; Masong et al., 2021; Mazigo et al., 2021, 2022a).

Standard treatment for FGS is a single dose administration of 40 mg/kg of praziquantel (McManus et al., 2018). However, mounting evidence indicates limitations of this treatment, particularly concerning its effectiveness in resolving the typical lesions caused by the disease (Norseth et al., 2014). The diagnosis of FGS is particularly challenging since the condition can be present also without living parasites actively excreting eggs. Thus, in the absence of FGS specific biomarkers, microscopy is not suitable for its diagnosis (Kjetland et al., 2014; WHO, 2015). Instead colposcopy through visual inspection of characteristic lesions, such as yellow sandy patches, abnormal blood vessels and rubbery papules, is designated as the standard diagnostic. Colposcopy is a relatively complex procedure (Norseth et al., 2014) that requires trained staff as well as appropriately equipped facilities (WHO, 2015). In resource limited settings (Mazigo et al., 2022b) these facilities are often not widely available and primarily concentrated at the tertiary levels of care (Xue et al., 2020). Nonetheless, the global lack of awareness surrounding FGS (Conseil National du Recensment de la Population et de l'habitation, 2021; Rasoamanamihaja et al.,

2023) adds complexity to its identification, even in high-income settings where infrastructure and professional capacity are typically not constraints (Rasoamanamihaja et al., 2023). Consequently, FGS diagnosis and treatment represents an UMN in both endemic and non-endemic contexts.

Madagascar is one of the countries with the highest prevalence of schistosome infections worldwide (Gruninger et al., 2023) with recent data showing also high prevalence of FGS (over 60%) (Kutz et al., 2023). In the country, there are no guidelines, which specifically pertain to the management of FGS (Rasoamanamihaja et al., 2023). The underlying assumption of our study is that FGS policies, guidelines and programs in Madagascar require (among others) awareness and knowledge of the disease. However, little is known about FGS awareness and knowledge among both women and HCWs in Madagascar. Our study aimed to address this gap by determining FGS knowledge among these two population groups in the highly *S. haematobium* endemic rural region of Boeny in Madagascar (Gruninger et al., 2023).

Methods

Study design, area and population

This cross-sectional survey study was conducted in the rural region of Boeny in Madagascar. Data were collected using a structured questionnaire administered by trained interviewers. The Boeny region has an estimated population of 543,200 inhabitants. Four municipalities within the region have been selected for the implementation of the study: Mahajanga (−15° 42′ 59.99″S-46°18′ 60.00″ E), Antanambao Andranolava (15° 57′ 59.99″S-46° 40′ 59.99″ E), Maravoay (−16° 06′ 38.30″ S-46° 38′ 37.79″ E) and Ankazomborona (−16° 06′ 60.00″S-46° 44′ 59.99″ E). The city of Mahajanga can be described as urban with 87,660 inhabitants (Conseil National du Recensment de la Population et de l'habitation, 2021), while the town of Marovoay with its 34,000 inhabitants can be classified as peri-urban. The two remaining study sites consist of the ten communities of Ankazomborona and eight communities of Antanambao-Andranolava with, respectively, 23,000 and 3,000 inhabitants corresponding to rural characteristics (INSTAT Madagascar - Institut National de la Statistique, 2022). The study sites have been selected according to variations in urbanicity, and the overall high estimated FGS prevalence of more than 60% in the region (Kutz et al., 2023).

Participant sampling, recruitment and eligibility criteria

Adult women from among the general population as well as male and female HCWs were selected using a convenience sampling approach. The total sample size was rounded to 1,000 as described in Conroy (2018), though, due to the COVID-19 pandemic, a smaller sample size was reached and a total of 820 individuals were surveyed. Participants were approached at markets, schools, health facilities, and their homes to assess their FGS awareness and knowledge. All participants were asked to sign an informed consent. In case of illiteracy an impartial witness was involved.

Inclusion criteria of the study were: (i) healthcare professionals working at the primary health care centres or female community members of Boney at markets, schools, health facilities or their home, (ii) older than 18 years of age, (iii) fluent in French and/or Malagasy, and (iv) willing and able to provide written informed consent.

Data collection and data management

Data were collected between 24/08/2020 and 04/09/2020. A paper-based questionnaire was administered and answered in either Malagasy or French. The questionnaire was structured into three thematic sections: (a) socioeconomic information, (b) awareness and knowledge of FGS, (c) health-seeking behaviour for non-medical personnel, and FGS treatment and diagnostic knowledge for HCWs. All questions were asked in a non-prompted format and if none of the pre-specified categories were applicable, additional answers were recorded in a free text/open response format.

All study participants were assigned a unique patient identifier (PID) to ensure data protection and pseudonymization. Questionnaires underwent a quality check following standard operating procedures. Double data entry was performed using the REDCap electronic data capture tools hosted at the Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany (Harris et al., 2009, 2019). Quality control of data processing and data validation was undertaken at regular intervals by data quality managers. The dataset was screened for missing values. Missing data corresponding with the exclusion criteria led to the exclusion of participants from the study. Missing values not leading to study exclusion were descriptively reported.

Statistical analysis

Descriptive statistics were calculated to summarize the socio-demographic characteristics of the population stratified by women and HCWs. Awareness of FGS was described through proportions and 95% confidence intervals (CI). The presence of awareness is assumed if a participant states that they have heard of FGS before today.

An FGS knowledge score was computed including five domains: (i) symptoms, (ii) transmission pathways, (iii) protective measures, (iv) consequences of the disease and (v) contribution of individuals to transmission. A maximum of 50 points was assigned to each participant, divided into 10 maximum points per domain. Both correct and incorrect answers were scored for each domain. Points were awarded for both answers mentioned correctly and unmentioned if correct. If no answers were ticked at all, 0 points were assigned. Open text answers were assigned to existing categories if possible. Score calculation is displayed in Supplementary Table S1. The overall score ranged from 0 to 50 points with the following categories: 0–20: no knowledge, 21–30: low knowledge, 31–40: medium knowledge, 41–50: high knowledge. Afterwards, sources of information were described through proportions for women and HCWs aware of FGS.

To calculate crude and adjusted prevalence ratios (CPR and APR) with 95% confidence intervals, a binary Poisson regression with robust standard errors was performed (Barros and Hirakata, 2003). FGS awareness was considered as dependent variable and age group,

urbanization of recruitment, place of interview, education and occupation as independent variables. The regression analysis was performed exclusively for female community members due to a limited sample size in the HCWs population. All statistics were performed using R version 4.2.2 (R Foundation for Statistical Computing, Vienna, Austria).

Ethical considerations

This study was approved by the Ethics Committee Hamburg State Medical Chamber (protocol number PV7309) and the National Ethics Committee of Madagascar (protocol number 052/MSANP/SG/AGMED/CERBM). No person who met the inclusion criteria was excluded as a participant in this study based on their sexual orientation, gender identity, political, ethnic affiliation, or socioeconomic position. Participants had the right to refuse to participate and to withdraw informed consent at any time without giving reasons. No financial incentives were given for study participation.

Results

Study population

A total of 93 HCWs and 727 women were surveyed. During the data cleaning process, 33 women were excluded due to the exclusion criterion of age less than 18 years. Missing age also resulted in exclusion from the study of 4 individuals. The final study population included in the analysis was then 93 HCWs and 690 women (Table 1).

The interviews were mostly conducted in Malagasy with the total of women surveyed in Malagasy, and just the 2.2% ($n = 2$) of the HCWs in French. Most common location of interviews for HCWs were, by design, health care facilities where 97.8% ($n = 91$) of HCWs were interviewed. In contrast, 83.2% ($n = 574$) of women were recruited in community settings such as participants homes or markets and 16.8% ($n = 116$) at health care facilities. The distribution of urban, peri-urban and rural study sites differed between women and HCWs. While among the women, 18.7% ($n = 129$) were surveyed in an urban setting, 41.2% ($n = 284$) were surveyed in a peri-urban and 40.2% ($n = 277$) in a rural setting, HCWs were most commonly interviewed in an urban setting [58.1% ($n = 54$)]. A total of 11.2% ($n = 12$) and 25.1% ($n = 27$) HCWs were interviewed in peri-urban and rural sites, respectively. The most represented age group among women was 18–25 years while among HCWs was 26–35 years.

The lowest level of education among HCWs was secondary education, reported for 4.3% ($n = 4$) of the population, while primary education or less among women was reported from 40.7% ($n = 281$) of the population. While 51.2% ($n = 352$) of the women indicated a secondary education, respectively 8.1% ($n = 56$) of the women and 94.6% ($n = 88$) of the HCWs had a university degree or completed vocational training.

Among women, 62.2% ($n = 429$) were farmers or fishers. Specific occupation was not recorded for HCWs. The majority of participants in both groups identified as Christian with 67.3% ($n = 505$) of the women and 90.3% ($n = 84$) of the HCWs. While none of the HCWs said they have no religion, 13.2% ($n = 91$) participants among the women indicated no religion.

TABLE 1 Socio-demographic characteristics of surveyed women and HCWs in the Boeny region of Madagascar.

	Women	Healthcare workers
	n (%)	*n* (%)
Total	690 (100)	93 (100)
Language of interview		
Malagasy	690 (100)	91(97.8)
French	0 (0)	2 (2.2)
Setting[a]		
Healthcare facility	116 (16.8)	91 (97.8)
Community	574 (83.2)	2 (2.2)
Age group		
18–25	247 (35.8)	27 (29.0)
26–35	224 (32.5)	29 (31.2)
36–45	117 (17.0)	23 (24.7)
46+	102 (14.8)	14 (15.1)
Urbanization		
Urban	129 (18.7)	54 (58.1)
Peri-urban	284 (41.2)	12 (11.2)
Rural	277 (40.2)	27 (25.1)
Education[b]		
Primary school and less	281 (40.7)	0 (0.0)
Secondary education	353 (51.2)	4 (4.3)
University/vocational training	56 (8.1)	88 (94.6)
Occupation[c]		
Non-farmer/-fisher	261 (37.8)	N/A
Farmer/-fisher	429 (62.2)	N/A
Religion[d]		
No religion	91 (13.2)	0 (0.0)
Christian	505 (67.3)	84 (90.3)
Muslim	24 (3.5)	6 (6.5)
Other	70 (10.1)	3 (3.3)

[a]Healthcare facilities are hospitals or primary health care centres (CSB), community is including markets, participants homes and other public places.
[b]"Primary school and less" includes no education; NA = 1 for HCW.
[c]Non-farmer/-fisher includes no occupation, employee, business, housewives, students and others (no answer, tailor, assistant or server).
[d]Other do include: do not know, no answer, divers sects.

Awareness of FGS in the study population

Among women, 11.3% [$n = 78$ (95% CI: 9.0–13.9)] were aware of FGS, compared to the 53.8% [$n = 50$ (95% CI 43.1–64.2)] of the HCWs (Figure 1). The 15.2% [$n = 34$ (95% CI: 10.7–20.6)] and 12.8% [$n = 15$ 95% CI: (7.4–20.3)] of women in the age groups 26–35 and 36–45, respectively, were aware of FGS, while lower proportions of FGS

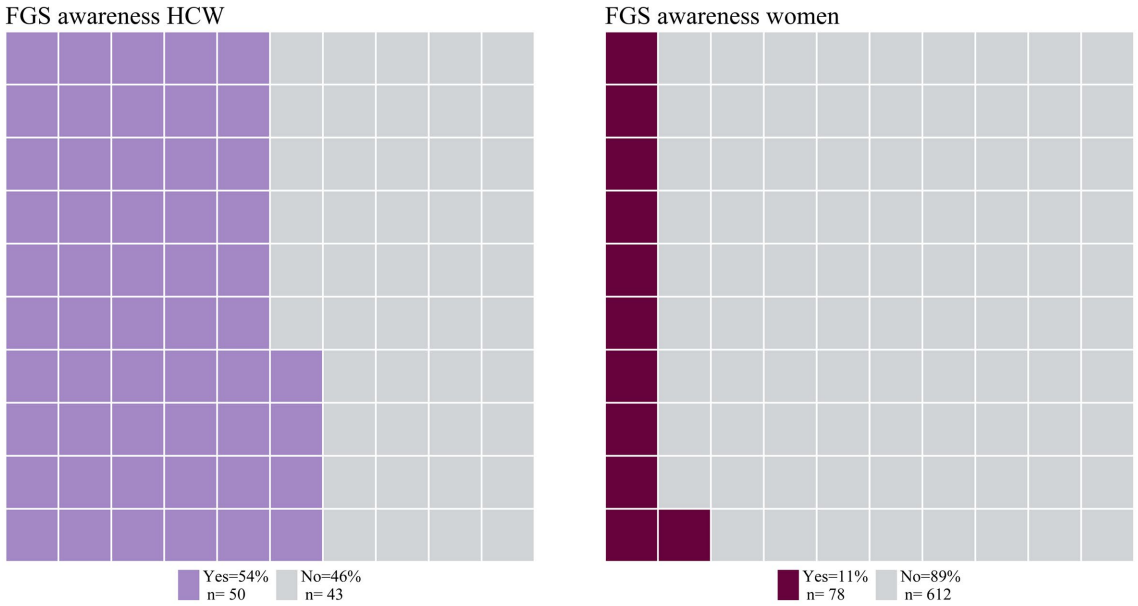

FIGURE 1
Awareness of FGS among HCW and women. Proportional waffle plot for *n* = 93 HCW and *n* = 690 women, one square representing 1% of the participants in this group.

awareness were observed in the age groups 18–25 [9.3%, *n* = 23 (95% CI: 6.0–13.6)] and 46+ [5.9%, *n* = 6 (95% CI: 2.2–12.4)]. The general awareness of FGS among women and HCWs are described in Supplementary Table S2.

Women living in urban areas had a higher awareness of FGS [24.0%; *n* = 31 (95% CI: 16.9–32.3)] as compared to women living in peri-urban or rural areas with 6.7% [*n* = 19 (4.1–10.3)] and 10.1% [*n* = 28 (95% CI, 6.8–14.3)] of awareness, respectively. Similarly, HCWs from the urban study site showed the highest level of general FGS awareness of FGS with 68.5% [*n* = 37 (54.4–80.5%)], while in the peri-urban and the rural locations lower proportions of the study population reported to be aware of FGS with 37.0% [*n* = 10 (19.4–57.6)] and 25.0% [*n* = 3 (5.5–57.2)] respectively.

A small difference in FGS awareness could be observed across occupations. While 13.8% [*n* = 36 (9.9–18.6)] of women not working as farmer or fisher were aware of FGS, only 9.8% [*n* = 42 (7.1–13.0)] of those working as a farmer or fisher showed awareness for the disease.

Knowledge score

Overall, it can be observed that among the 690 women, 9.3% [*n* = 64 (95% CI 7.2–11.7)] reached low knowledge scores, 1.9% [*n* = 13 (95% CI 1.0–3.2)] medium and 88.8% [*n* = 613 (95% CI 86.3–91.1)] had no knowledge. In total 47.3% [*n* = 44 (95% CI 36.9–57.9)] of HCWs had low knowledge of FGS, 6.5% [*n* = 6 (95% CI 2.4–13.5)] had medium knowledge and 46.2% [*n* = 43(95% CI 35.8–56.9)] had no knowledge of FGS. Neither the women nor the HCWs included in this study reached a high knowledge score.

The knowledge score for participants aware of FGS prior to the survey is displayed in Figure 2. While HCWs had a slightly higher median score of 28 (IQR: 25–29) with a minimum score of 21 and a maximum score of 32, women had a higher maximum score with a median knowledge score of 27 (IQR: 26–29) with a minimum score of 20 and a maximum score of 34.

Sources of information

Among the 76 women and 50 HCWs who reported awareness of FGS, the source of information about the disease in the last 6 months was investigated and summarised in Figure 3. Family [32.1% (*n* = 25)] as well as midwives and the radio with each 11.5% (*n* = 9) represent the most common sources of information for women.

Information about FGS among HCWs is mostly acquired through other HCWs [40% (*n* = 20)]. School curricula were mentioned by 10% (*n* = 5) of the HCWs as their source of information. Among the HCWs (*n* = 50) and women (*n* = 76) aware of FGS, 36% (*n* = 18) and 26.9% (*n* = 21) respectively mentioned that they did not receive any information in the 6 months prior to the survey. Other less frequently named sources of information were awareness campaigns or community workers for the women, and television for the HCWs.

Prevalence ratio for FGS awareness among women

CPR and APR were estimated for associations with variables considered as possible influencing factors for FGS awareness. The model was performed exclusively among the women (*n* = 690) because of the limited sample size (*n* = 93) of HCWs. CPR and APR for FGS awareness among women are listed in Supplementary Table S3.

The adjusted model displayed in Figure 4 shows a non-significant residual deviance and a moderate variance inflation factor ranging from 1.1–1.4 for all predictors.

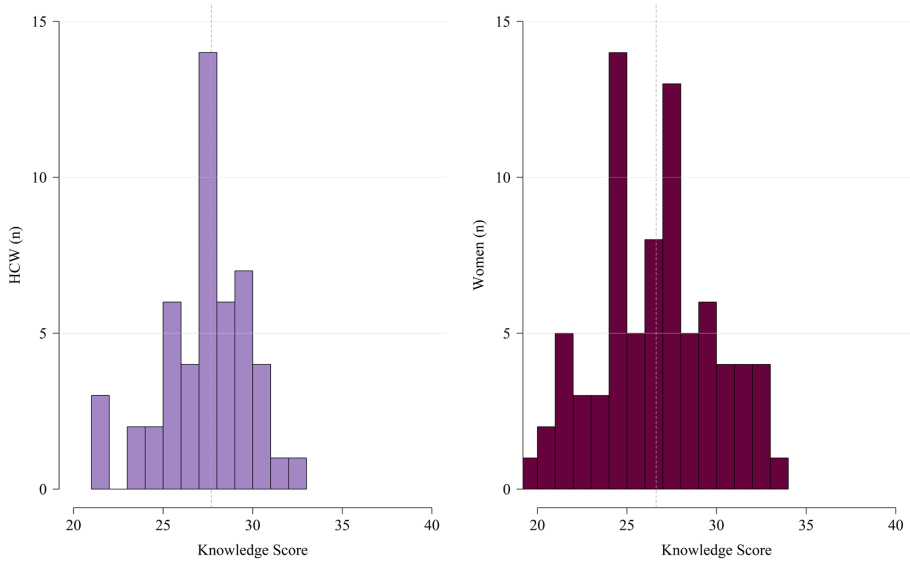

FIGURE 2
Distribution of knowledge score among HCWs (*n* = 50) and women (*n* = 78) with previous awareness of FGS. Dashed line is representing the median.

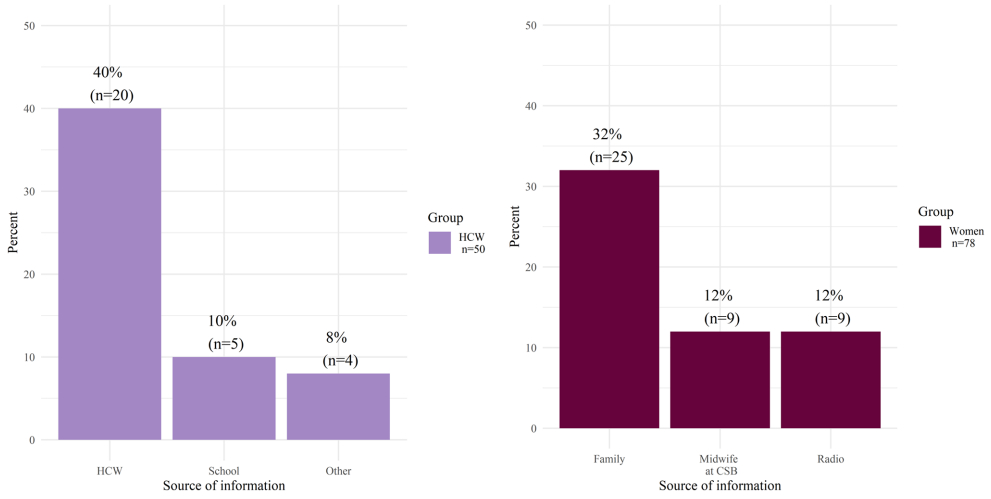

FIGURE 3
Three most common sources of FGS information 6 months prior to the survey for HCWs, and for women.

Peri-urban locations [APR 0.26 (95% CI: 0.15; 0.45)] and rural locations [APR 0.37 (95% CI 0.22; 0.63)] had shown a reduced prevalence, statistically significant, of 74% and 63% FGS awareness respectively, when compared to the urban location of Mahajanga.

Women who attended university or completed a vocational training had twice the prevalence, statistically significant, of FGS awareness when compared to the group with a primary education or less [APR 2.0 (95% CI 1.00; 4.00)]. However, no difference was found when comparing the prevalence of FGS knowledge among women with secondary education and primary education or less [APR 0.96 (95% CI 0.58; 1.60)].

An increased prevalence of FGS awareness was observed when comparing the location of the interview in a community setting to a location of the interview in a hospital setting [APR 1.61 (95% CI 0.87; 2.97)]. Furthermore, an increased APR was observed for the age groups 26–35 [APR 1.55 (95% CI 0.94; 2.56)], 36–45 [APR 1.56 (95% CI 0.85; 2.88)] and 46+ [APR 0.68 (95% CI 0.28;1.67)] when compared to the age group 18–25. No difference in the prevalence of awareness between farmer/-fisher and non-farmer/-fisher was found [APR 1.00 (95% CI 0.63, 1.58)].

Discussion

Limited FGS awareness and knowledge can result in UMN related to FGS. Our study shows overall low awareness and knowledge of the

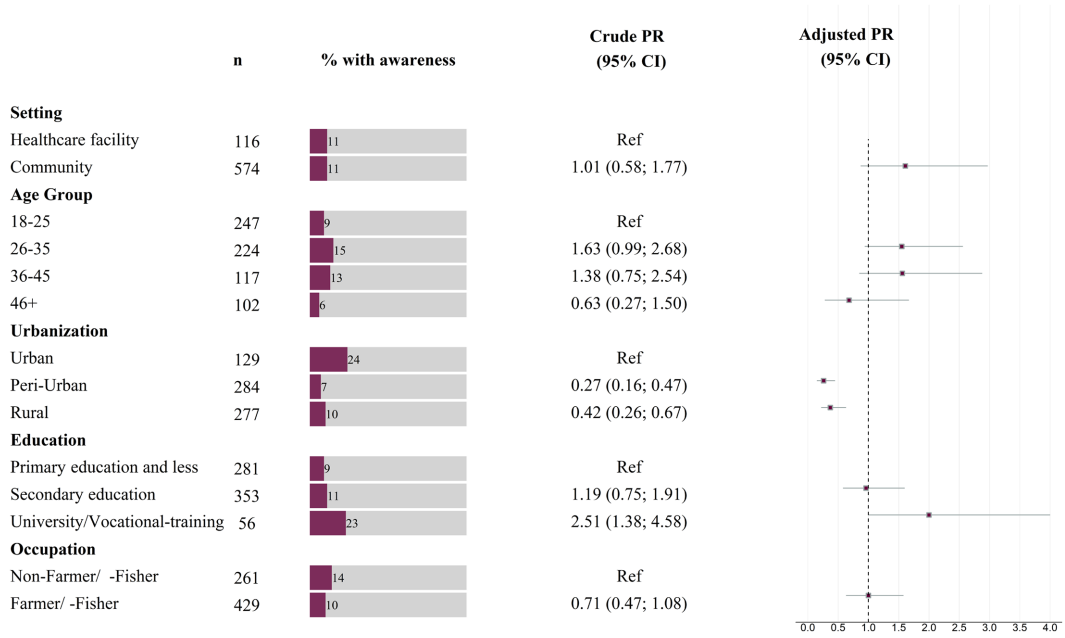

FIGURE 4
Prevalence ratios for FGS awareness among women (n = 690), based on binary Poisson regression models with robust standard errors. Box-whisker plot indicates adjusted prevalence ratio and 95% confidence interval.

disease in the highly endemic context of the Boeny region in Madagascar, and contributes to identifying an important health gap, which relates to a disease that can silently affect millions of women worldwide.

The main findings of our study show that 54% of HCWs and 11% of women were aware of FGS while both HCWs and women have limited knowledge of the diseases according to the score established and computed in this study. We report that living in an urban area [non-urban settings: APR 0.26 (95% CI: 0.15; 0.45); APR 0.37 (95% CI 0.22; 0.63)] and having completed university education or vocational training [APR 2.0 (95% CI 1.00; 4.00)] are factors influencing FGS awareness in women. Interestingly, we observe that those working as farmers, commonly reported to be at more at risk for schistosomiasis (Gruninger et al., 2023), are slightly less aware of FGS even though, from the model, we cannot conclude that this factor directly influences awareness. Finally, we report that in both groups, the most common sources of FGS knowledge were peers: other HCWs among HCWs and family members among women. Our main findings are aligned with existing literature from other endemic countries (Kukula et al., 2019; Mazigo et al., 2021, 2022b) and with an exploratory and qualitative study conducted in Madagascar in 2022, on 76 women in the Ambanja district (Schuster et al., 2022).

Schistosomiasis is a high burden disease particularly in SSA countries, which is mostly being addressed with MDA programs (French et al., 2018). This has generated a general awareness and knowledge of the disease among policy makers, HCWs, the at-risk populations and other stakeholders (Christinet et al., 2016). MDAs have been promoted and implemented with the intent of controlling the disease by decreasing its transmission in highly endemic areas (Kokaliaris et al., 2022). Unfortunately, since this strategy has not shown the expected public health impact so far (Li et al., 2019) there is the distinct possibility of an increase in chronic forms of the disease, which remain mostly undetected and untreated (Christinet et al., 2016). Our findings show that in a context that is highly endemic for FGS (Kutz et al., 2023), awareness and knowledge of the disease among both users and providers of health services are low. This may be one important reason for the notable lack of both supply and demand of health services for the detection and management of the FGS in the country (Rasoamanamihaja et al., 2023). Neglecting chronic forms of schistosomiasis, such as FGS, not only contributes to an increasing disability-adjusted life years (DALYs), decreasing quality adjusted life years (QALYs), and perpetuating the vicious cycle of poverty for affected populations, but also to the transmission of the infection in highly endemic areas. The 2021–2030 WHO NTD roadmap (World Health Organization, 2021) addresses the elimination of schistosomiasis as a public health problem by 2030. If immediate actions to address the chronic forms of the disease are not taken, the WHO target will be hard to reach. Chronic forms of schistosomiasis require a paradigm shift in the management of the disease, moving away from conventional mass strategies within vertical programs. Instead, the focus should be on integrated services that can cater more to the needs of affected individuals.

Furthermore, limited awareness and knowledge of FGS can contribute to exacerbate gender gaps and social inequalities among vulnerable populations. FGS presents signs and symptoms that can be similar to/misinterpreted as being sexually transmitted diseases (STDs) (Mazigo et al., 2022b). Despite the progress made in the control and management of STDs worldwide (Zheng et al., 2022), social stigma still remains one of the major problems associated with these diseases (Lee and Cody, 2020; Mazigo et al., 2022b). The major consequence of social stigma is the fear of the population, especially among women, of using health services (Rusch et al., 2008) due to the consequent marginalisation that they could experience in their communities (Avuvika et al., 2017). Additionally, a lack of knowledge

of the FGS among HCWs can lead to incorrect diagnosis (Mazigo et al., 2022b) and treatment (WHO, 2015). Raising awareness and knowledge about FGS, could help encourage women to seek timely medical care, prevent long term consequences, such as infertility or cancer associated with the disease (Mazigo et al., 2022a), and reduce antimicrobial resistance due to inappropriate use of antibiotics (Nemungadi et al., 2022).

Notably, our data show that both HCWs and women in the general population report peers as being their main source of information about the disease. Peer communication among patients has proven to be effective in increasing awareness and knowledge of different diseases ranging from HIV (Ayala et al., 2021) to cancer (Ancker et al., 2009), and COVID-19 (Shoghli et al., 2023). Various health strategies have already been conceptualised, implemented and frequently integrated at different levels of care depending on the type of disease and context (Ancker et al., 2009; Markowski et al., 2021). For instance, peer leaders in the fight against HIV have been involved in several health programs in highly endemic countries, such as South Africa (Ayala et al., 2021), not only to raise awareness about the disease but also to promote health services, such as prevention and screening in rural communities (Mannoh et al., 2022). Similarly, for FGS, the involvement of community leaders in health communication campaigns could improve health seeking behaviour of the affected populations. Unfortunately, in the absence of an easy, field applicable diagnostic, FGS community-based screening programs must rely on highly equipped health facilities, which makes the design and implementation of widely accessible and sustainable services challenging (Xue et al., 2020).

Additionally, our findings show that those with higher education (APR 2.0 [95% CI 1.00; 4.00]) and those living in urban settings [non-urban settings: APR 0.26 (95% CI: 0.15; 0.45); APR 0.37 (95% CI 0.22; 0.63)] have a higher prevalence of FGS awareness. In Madagascar, most of the population lives in remote areas and more than 5 km away from health care facilities (U.S. Agency for International Development, 2022), which represents a major challenge to the provision of access to care and to (health) education (Rasoamanamihaja et al., 2023). In general, FGS is rarely mentioned in medical textbooks nor a topic covered as part of continuing medical education (CME) programs (UNAIDS, 2019). The lack of FGS-related CME in combination with a lack of routine services and thus little exposure of HCWs to direct medical practice for FGS, further complicates the translation of theory into practice (Gupta et al., 2017). In fact, the implementation of CME programs is challenging in Madagascar not only for the common barriers encountered in LMICs (Merry et al., 2023), such as budget limitations or scarcity of equipment (English et al., 2016), but also due to the scarcity of medical personnel in health facilities who cannot be readily replaced in their absence (U.S. Agency for International Development, 2022).

This study provides valuable insights on the under-researched topic of FGS awareness and knowledge among a comparatively large sample of women and HCWs in a highly endemic country. Moreover, it introduces the concept of a score to assess knowledge of the disease, which helps to identify specific gaps among health care users and providers, which need addressing. Finally, its findings provide elements for the design and implementation of FGS-related awareness campaigns and training, including among others the use of peer education. Despites its strengths, our study is not without limitations. Firstly, this study used a convenience sampling approach, which can have implications in terms of the generalizability from the enrolled population to the wider two target populations of both women from the general population and HCWs. Further, our data are drawn from a cross-sectional survey, meaning that there are limitations to the interpretability of risk associations. Since recruitment took in part place at health care facilities, which had been previously part of other schistosomiasis-related research projects, a bias may have been introduced, which means that an overestimation of awareness and knowledge among participants cannot be excluded. Furthermore, the conceptualization of the knowledge score was specifically designed to fit the context of the study. Even though it is informed by other existing tools, ours is not validated. Due to the structure of the data collection tool, we cannot report differences in the level of knowledge segregated by different professions of the health care system (i.e., doctors, nurses, midwives, community health care workers) nor address age among specific groups (i.e., reproductive age). Moreover, the high proportion of Christians within our sample does not allow to make any conclusion about variations across religious groups and possible differences in social norms and behaviours in terms of access to and use of health services (Kang et al., 2020). Finally, in our study we exclusively address women, since they are the ones directly affected by FGS. Further research is needed to account for the role of men in the possible transfer of and access to FGS awareness and knowledge in the communities within this setting.

Conclusion

In conclusion, our study identifies important gaps in FGS-related knowledge among affected women and healthcare workers in Madagascar. In alignment with the WHO NTD Road Map, this has important implications for the control and management of schistosomiasis, and its more chronic forms. Raising awareness and knowledge of chronic forms of NTDs can help address diseases that can silently affect individuals worldwide.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethics Committee Hamburg State Medical Chamber (protocol number PV7309) and the National Ethics Committee of Madagascar (protocol number 052/MSANP/SG/AGMED/CERBM). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

PR: Data curation, Formal analysis, Methodology, Visualization, Writing – original draft. RAR: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. RR: Project administration, Supervision, Writing – review & editing. RSR: Supervision, Writing – review & editing, Investigation. SR: Investigation, Supervision, Writing – review & editing, Project administration. TR: Conceptualization, Funding acquisition, Investigation, Project administration, Writing – review & editing. J-MK: Investigation, Project administration, Supervision, Writing – review & editing. AJ: Data curation, Writing – review & editing. YH: Investigation, Writing – review & editing. EL: Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – review & editing. JM: Resources, Writing – review & editing. DP: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. DF: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1278974/full#supplementary-material

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Group B Streptococci recto-vaginal colonization, antimicrobial susceptibility pattern, and associated factors among pregnant women at selected health facilities of Wolaita Sodo Town, Southern Ethiopia

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Background: *Streptococcus agalactiae* or *Group B Streptococcal* colonization of the gastrointestinal and genital tracts of pregnant women usually remains asymptomatic, even though it is the critical determinant of infection in neonates and young infants. It causes early and late onset of invasive *Group B Streptococcus (GBS)* disease manifesting as septicemia, meningitis, and pneumonia. Now it is recognized as an important cause of maternal and neonatal morbidity and mortality in many parts of the world including Ethiopia, where the magnitude of the problem has been little studied. The aim of this study was to assess the prevalence of *GBS* colonization and to identify associated risk factors and antimicrobial susceptibility patterns among pregnant women at selected health facilities of Wolaita Sodo Town, Southern Ethiopia.

Methodology: A health-facility-based cross-sectional study design was conducted at WSUCSH & Wolaita Sodo Health Center from June to August, 2022. A total of 279 pregnant women who were in ANC follow-up at 35–37 weeks of gestation were included. For *GBS* isolation, recto-vaginal swabs were inoculated in 1 mL Todd-Hewitt broth medium supplemented with 10 μg/mL colistin and 15 μg/mL nalidixic acid, followed by identification of isolates based on colonial morphology, gram stains, catalase reaction, and CAMP tests. Antimicrobial susceptibility testing was performed using a modified Kirby–Bauer disc diffusion method. All collected data were entered in Epi info 4.6.0.2, then transferred and tabulated using SPSS version 20. Logistic regression analysis was used to see the association between variables. Finally, a p-value <0.05 was considered statistically significant.

Results: In the present study, 279 pregnant mothers, aged between 15 to 38 years with a mean of 26.5 ± 4.5 years, were included. Of all participants, the highest proportion (120) (43.01%) were housewives. The overall carriage rate of *GBS* was 67 (24.0%). *GBS* colonization showed a statistically significant association with college and above levels of maternal education [AOR = 6.610, 95% CI (1.724–

25.349), $p = 0.01$]. High susceptibility of *GBS* isolate was seen with Penicillin G & Chloramphenicol (92.5%), Ampicillin, Ceftriaxone (89.6%), Vancomycin (74.62%), and Erythromycin (77%). Relatively, *GBS* showed high resistance to Tetracycline (88%).

Conclusion and recommendation: In this study, the overall prevalence of *GBS* colonization was 24.0%. College and above educational level was statistically significant with *GBS* colonization. This study aimed to draw attention to the management of *Group B Streptococci* in pregnant women by making *GBS* culture one of the routine diagnoses during ANC follow-up and to prevent infection with early detection.

KEYWORDS

***GBS*, antibiotic susceptibility pattern, pregnant women, Wolaita Sodo, Ethiopian**

1. Introduction

Streptococcus agalactiae (GBS) is a typical microbiota of healthy adults' female genital tracts and anal areas, with the gastrointestinal tract acting as a natural reservoir and source of vaginal colonization. Pregnancy-related diseases such as urinary tract infection, bacteremia, chorioamnionitis, postpartum endometritis, preterm labor, preterm rupture of membranes, and perinatal transfer of the organism are all possible outcomes of maternal *GBS colonization. GBS's* ability to rise from the lower genital tract and colonize the upper genital tract has been linked to intrauterine infection (Lämmler et al., 1995; Mengist et al., 2016, 2017; Liesse Iyamba et al., 2021).

Group B Streptococcus colonization of the genitourinary or gastrointestinal tract of pregnant women and its transmission to the infant during the labor and delivery process is the principal risk factor for early onset invasive *GBS* disease (Dadi et al., 2022). During pregnancy, approximately 10–30% of pregnant women are colonized with *GBS* in the vaginal and rectum area asymptomatically and 60% of their babies are infected through the birth canal (Melin and Efstratiou, 2013; Dadi et al., 2022).

Antibiotic resistance among *GBS* isolates has also been a concern due to the extensive use of intrapartum antibiotic prophylaxis to avoid early-onset *GBS* illness (Mengist et al., 2016, 2017; Leykun et al., 2021). *Streptococcus agalactiae* remain fully susceptible to penicillin as well as to most β-lactams, and penicillin remains the first-choice antibiotic to prevent *GBS*-EOD and to treat *GBS* disease. However, over the last two decades, resistance to macrolides and clindamycin among invasive isolates of *GBS* has increased from <5% to common resistance of 20–30% (Melin and Efstratiou, 2013; Dadi et al., 2022).

Group B Streptococcus colonization of pregnant mother is different in different countries. In the United States and Europe, *GBS* is the major cause of mortality and morbidity. It can be found in the vaginal microbiota of up to 30% of pregnant women and can be transmitted to the infant via a perinatal transmission (Centers for Disease Control and Prevention, 2007). A review that has specifically looked at the prevalence of maternal colonization with *GBS* indicated the estimated mean prevalence of *GBS* colonization was 17.9% overall and was the highest in Africa (22.4%) followed by the Americas (19.7%) and Europe (19.0%). However, studies from Southeast Asia had the lowest estimated mean prevalence (11.1%) (Kwatra et al., 2016; Mengist et al., 2016; Nishihara et al., 2017). Reduced by almost 80% in the United States, cases fell from 1.8 cases per 1,000 live births in the early 1990s to 0.23 cases per 1,000 live births in 2015 (Mengist et al., 2016). Evidence on maternal colonization prevalence remains sparse in African settings (Gizachew et al., 2019). In sub-Saharan Africa, the prevalence of *GBS* in Kampala, central Uganda was 3.9% (Tumuhamye et al., 2021), in the Democratic Republic of Congo, 23.07% (Liesse Iyamba et al., 2021), and in Kenya 20.5% (Jisuvei et al., 2020). In Sri Lanka, *GBS* vaginal colonization was 18% (Dilrukshi et al., 2021). Specifically in Ethiopia, studies have revealed maternal colonization ranges from 7.2% (Woldu et al., 2014) to 25.5% (Gizachew et al., 2019). Different studies conducted in Ethiopia indicated that Addis Ababa's prevalence of *GBS* colonization among pregnant women was 14.6% (Assefa et al., 2018), in Jimma the prevalence of *GBS* colonization among pregnant women was 19.0% (Mengist et al., 2016), in Gondar the prevalence of *GBS* colonization among pregnant women was 25.5% (Gizachew et al., 2019), and in Nekemte the prevalence of *GBS* colonization among pregnant women was 12% (Mengist et al., 2017). Significant differences in the frequency of maternal colonization have been reported according to region, ethnicity, and socioeconomic characteristics (Kfouri et al., 2021).

Worldwide mortality from *GBS* colonization decreased from 12.7 million in 1990 to 6.3 million in 2013, but continuous effective measures should be made to decrease the mortality of newborns in developing countries (Nishihara et al., 2017). In Africa, the mortality rate is 4 times higher compared to America and Europe. So strategies for the prevention of *GBS* have a crucial role in mortality (Centers for Disease Control and Prevention, 2002; Nishihara et al., 2017).

GBS has the potential to thrive in a variety of diverse host environments (Plainvert et al., 2021). The problem is particularly immense in developing countries like Ethiopia that do not have quality microbiological laboratory facilities to isolate pathogens and determine their antimicrobial susceptibility pattern, in addition to the presence of fake drugs in circulation, and misuse of antimicrobials by health care providers, unskilled practitioners, and patients. Effective use of intrapartum antibiotic prophylaxis (IAP) reduces around 80% of early onset *GBS* disease. So a strategy on IAP evaluation for the prevention of EOD should be done in developed countries to decrease the burden of *GBS* disease, to develop a vaccine, or to prepare another preventive plan (Nishihara et al., 2017). There are many studies

conducted in different cities of Ethiopia that show a high prevalence of the disease in mothers. However, there is no strategic plan developed to minimize the disease.

GBS infection is a challenging problem; much research has been done to show the prevalence of *GBS*, and its antimicrobial pattern has changed from place to place and from time to time. So the epidemiological data needs to be updated for a given place and time (Arain et al., 2015). Therefore, the main aim of this study will be to determine the prevalence of *GBS* bacteria in pregnant women and to carry out an antimicrobial susceptibility test in Wolaita Sodo Town, Southern Ethiopia. The result of this study may show the currently updated burden of the disease in Wolaita Sodo Town, Southern Ethiopia. This study could provide updated information for responsible bodies to formulate policies to implement prevention plans through universal screening for *GBS* in ANC units and effective use of prophylaxis to prevent early *GBS* infection.

2. Materials and methods

2.1. Study area

The study was conducted in Wolaita Sodo Town, which is located 327 km from Addis Ababa and 129 km from Hawassa. There are two general public hospitals, one governmental specialized hospital (WSUCSH), and three government health centers. In this study, two health facilities were used: WSUCSH and Wolaita Sodo Health Center (WSUCSH Co, 2022).

Wolaita Sodo Health Center under SNNPR Health Bureau provides outpatient services (ANC), follow-ups for adult OPD, pediatric OPD, a delivery service, TB patient follow-up, HIV counseling and screening, and health package services.

WSUCSH is a teaching and referral hospital of Wolaita Sodo University Health College, which started community service in 2009. It has about 500 beds, more than 300 health workers, and serves an average of about 1,000,000 patients annually (WSUCSH Co, 2022).

2.2. Study design and period

A health-facility-based cross-sectional study design was conducted at the ANC clinic of Wolaita Sodo University Comprehensive Specialized Hospital and Wolaita Sodo Health Center from June to August 2022.

2.3. Study population

All pregnant mothers who attended ANC follow-up at WSUCSH and Wolaita Sodo Health Center in Sodo Town and who were in their 35 to 37 weeks of gestational period were part of the study population.

2.4. Sample size determination

Sample size was determined using single population proportion formula considering the following assumptions: 95% confidence interval, 5% margin of error, and an assumed prevalence of 20.9% from the prevalence of *GBS colonization* among pregnant women in a previous study done Hawassa by Mohammed et al. (2010).

The following standard formula was used to calculate it.

$$n = \frac{Z\alpha^2 P(1-P)}{d^2}$$

By using a 95% confidence level, the Z value was 1.96, with a 5% margin of error (d).

P = estimated prevalence rate = (20.9%); α = 0.05 (level of significance).

n = the required sample size.

$$n = \frac{(1.96)2(0.209[1-0.209])}{(0.05)^2} = \frac{3.8416*(0.209*0.791)}{0.0025}$$

$$= 254 + (10\%\text{Non} - \text{response rate}) : 254 + 25 = \underline{\underline{\mathbf{279}}}$$

2.5. Sampling method

The study participants were enrolled using a systematic sampling technique until a sample size of 279 was achieved. The first participant was selected by the lottery method and by using the formula $K = N/n$ ($K = 424/279 = 1.52 \sim = 2$ from Wolaita Sodo Health Center and $K = 652/279 = 2.3 \sim = 2$ WSUCSH) over a three-month period. Therefore, individual participants were selected randomly at every Kth interval from two data collection institutions during the study period.

Then, the final study population of 279 was allocated proportionally to the size for each health facility based on their pregnant women visit as shown below. Pregnant women ANC visit in the year of 2021 from both Health facility data were shown below.

- n_{WSUCSH} = number of sample participants required from Wolaita Sodo University Comprehensive Specialized Hospital.
- N_{WSUCSH} = number of pregnant women who came to WSUCH from June 1, 2021 to August 30, 2021 GC, which was 652.
- n_{WSHC} = number of sample participants required from Wolaita Sodo Health Center.
- N_{WSHC} = number of pregnant women who came to Wolaita Sodo Health Center from June 1, 2021 to August 30, 2021, which was 424.
- n = is the total sample size of the study, which was 279.
- N_{Total} = the sum of pregnant women both facilities from June 1 2021 to August 30, 2021, which was 1,076.

Therefore,

- n_{WSCSH} (from Wolaita Sodo Comprehensive Specialized Hospital) = $(n/N_{Total}) - N_{WSUCSH}$.
- $n_{WSUCSH} = (279/1076) \times 652 = 169$, sample size was allocated to WSUCH.

Whereas

- n_{WSHC} (from Wolaita Sodo Health Center) = $(n/ N_{Total}) \times N_{WSHC}$.
- $n_{WSHC} = (279/1076) \times 424 = 110$ sample size was allocated to $_{WSHC}$.

2.6. Data collection

The data on socio-demographic variables and other relevant information were collected using a predesigned and pretested structured questionnaire and by reviewing medical records. The questionnaire was adapted from other similar studies and initially prepared in English, translated to Amharic, and then translated back to English by another translator to check for consistency. Informed consent was obtained from each study participant after explaining the purpose and procedure of the study. The questionnaire was administered by the attending midwives and nurses to pregnant women with a gestational age between 35 and 37 weeks.

2.6.1. Specimen collection

Specimens were collected as per the ACOG committee opinion and American Society for Microbiology (ASM) protocols. A vaginal-rectal swab was sampled from the mother at the point of ANC and labor by trained midwives using a sterile cotton swab. Using an aseptic technique by applying sterile cotton-tipped swabs in separate sterile tubes at the site of the rectum and vagina, the vagina swab from the mucosal secretions of the lower-third part was obtained. Thereafter, the rectum swab was carefully inserted into the anal sphincter and gently rotated to touch the anal crypts. Within 30 min the vaginal swab was placed in Amies transport media and within an hour of collection was transported to the Microbiology Laboratory of WSUCSH. Samples were transported in an ice box. All samples were cultured within an hour of arrival at the laboratory following standard bacteriological techniques (Leykun et al., 2021).

2.7. Laboratory procedures

2.7.1. Culture and identification of *Group B Streptococci*

The swabs were inoculated in 1 mL Todd-Hewitt broth, an enrichment medium for *GBS*, supplemented with 10 µg/mL colistin and 15 µg/mL nalidixic acid to prevent contaminant growth. The samples were then incubated at 37°C aerobically for 18–24 h, sub-cultured onto sheep blood agar plates, and re-incubated at 37°C. After 24 h, the cultures were inspected for growth and all negative culture plates re-incubated for an additional 18–24 h and then re-observed. Plates that showed growth were identified by their characteristic appearance and biochemical tests such as catalase and CAMP testing; those with no growth were discarded or reported as negative (Figure 1).

CAMP testing was performed on sheep blood agar plate (SBAP) by streaking of *S. aureus* down the middle of SBAP and the test organism was then streaked perpendicularly to the *Staphylococcal* streak. The streaks did not touch. CAMP factor produced by *S. agalactiae* and β lysine produced by *S. aureus* act synergistically on SBAP to produce enhanced hemolysis. After incubation overnight under candle jar atmospheres, the SBAP was examined for an arrowhead-shaped zone of enhanced lysis Christie, Atkins, and Munch-Petersen (CAMP) factors. Those that were Gram-positive cocci in gram stain, catalase-negative in Biochemical tests, and CAMP positive were identified as *S. agalactiae* (Shiferawu et al., 2019).

2.7.2. Antimicrobial susceptibility testing

Kirby Bauer's disc diffusion technique was used to test the Antibiotic susceptibility (AST). The media used was Muller Hinton agar (MHA) supplemented with 5% sheep blood. From a fresh non-selective agar plate, pure colonies were selected and transferred to 5 mL Sterile normal saline and thoroughly mixed to make the suspension homogeneous. Turbidity was adjusted using a McFarland densitometer to match with a 0.5 McFarland Standard, then inoculated following the standard over the entire surface of an MHA plate using a sterile swab. Then, using sterile forceps, the antibiotic disks were placed on MHA 15 mm from the border and by considering the 24 mm distance between each disc and Zone of Inhibition was measured by the metric scale and reported as susceptible (S), intermediate (I), or resistance (R). Using the updated guidelines (CLSI, 2021), the following antibiotics disks were used for *Group B Streptococcus susceptibility*: Penicillin G 10 IU, Ampicillin 10, Erythromycin 15 Clindamycin 2, Ceftriaxone 30, Ciprofloxacin 5, chloramphenicol 30, Clindamycin 2, Vancomycin 30, and Tetracycline 30.

2.8. Data quality control

To assure the quality of the data, a pre-test was done and 5% of the total sample was out of the study area. Training was given to the data collectors on interviewing and recto-vaginal swab sample collection. Laboratory attendants were trained on how to clean, sterilize, and reuse laboratory materials. Investigators were trained on how to collect recto-vaginal swab samples by trained data collectors. The specimens were transported to the WSUCSH Central Laboratory within 30 min of collection in a cold chain (ice-box at 4°C) and immediately processed. Inoculums density for bacterial suspension for the antimicrobial susceptibility testing was standardized to 0.5. McFarlane Supervision was undertaken during the whole phase of the study period by the investigator and Medical Microbiologist. All culture media was prepared following the manufacturer's instructions. All media was checked for sterility and performance. Reference strain *S. aureus* (ATCC-25923) was used as quality control throughout the study for culture and antimicrobial susceptibility testing. *E. fecalis* (ATCC-25212) and *S. pyogenes* (ATCC 19615) were used as a negative control for CAMP testing. To check the quality of the culture media and antimicrobial disks, control organisms were obtained from the EPHI (Ethiopian Public Health Institute). Samples were collected and processed aseptically using a standard operating procedure.

2.9. Methods of data analysis

Data were entered, cleaned, and processed into Epi 4.6.0.2 using SPSS version 20. Logistic regression analysis was used to see the association between variables. The "*p*" value was less than 0.05, which was considered statistically significant.

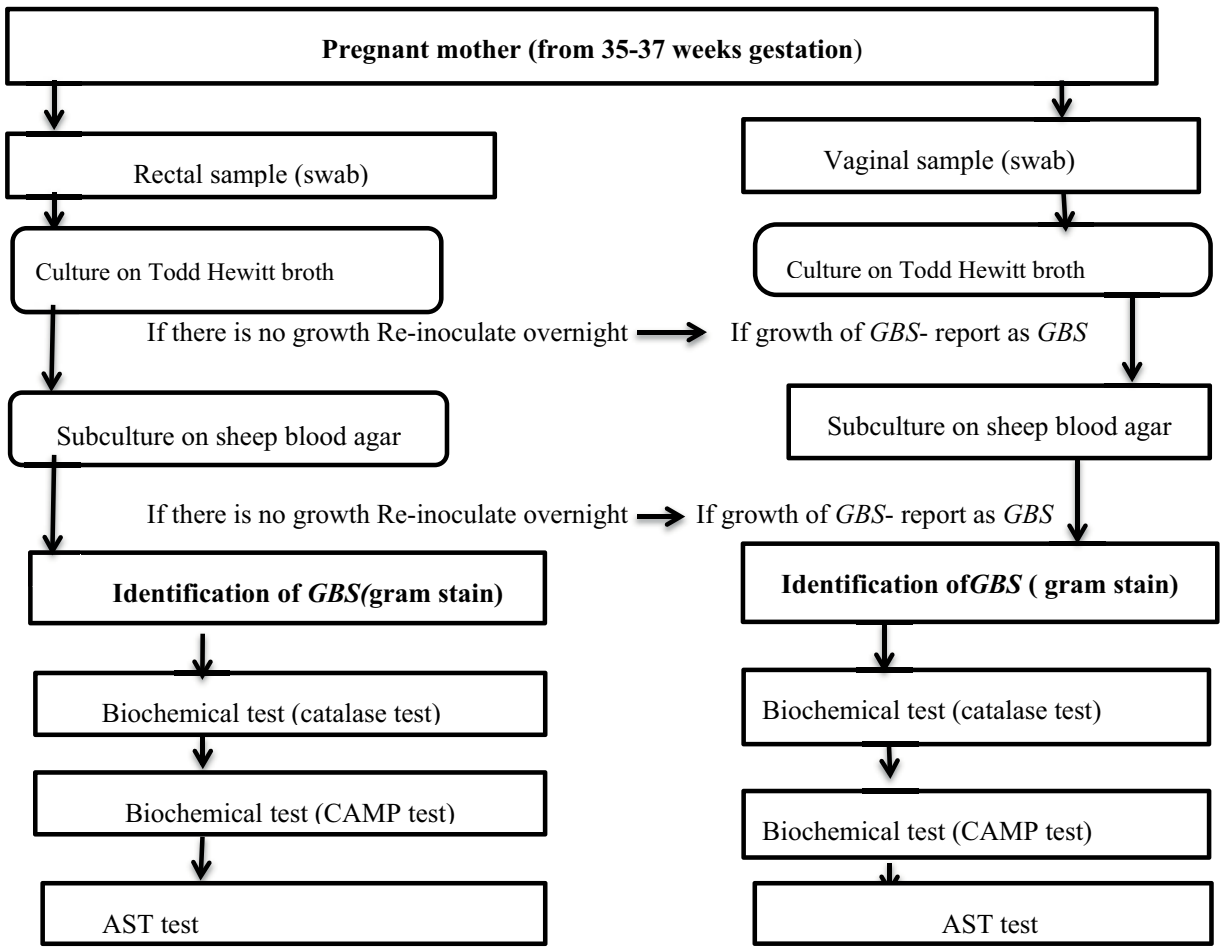

FIGURE 1
Flow chart diagram showing culture isolation, laboratory identification, and AST of *GBS*.

3. Results

3.1. Socio-demographic characteristics

A total of 279 pregnant women (from 35 to 37 weeks of gestation) were enrolled from June to August 2022, with a total response rate of 100%. The age of the study participants was between 15 to 38 years, with a mean age with SD of 26.5 (± 4.5) years. Most of the study participants (135) were aged between 25 and 29 years (48.4%). The majority of the study participants were married (249) (89.2%) and were urban residents (260) (93.2%). Of all participants, the highest proportion (120) (43.01%) were housewives, followed by civil servants (64) (22.9%), students (Garland et al., 2000) (17.9%), and merchants (Fatemi et al., 2009) (16.0%). A socio-demographic characteristic of study participants is given in Table 1.

3.2. Obstetric and clinical characteristics

Regarding obstetric and clinical characteristics of study participants, 173 (62.0%) were primigravida and the remaining 106 (38%) were multigravida. Of the study participants, 51 (18.3%) had a history of abortion and 13 (4.7%) women had a history of preterm labor. Among the 279 pregnant women included in the study, 58 (20.8%) were at a gestational age of 35, 146 (52.3%) were at a gestational age of 36, and the rest (75) (26.9%) were at 37 weeks of gestational age. History of hormonal contraceptive usage was reported by 223 (79.9%) of the study participants (Table 2).

3.3. *Group B Streptococcus* colonization

The overall prevalence of *GBS* colonization among pregnant women at 35–37 weeks of gestation was 24% (67/279). The prevalence of *GBS* in the two health institutions was 37/67 (55.2%) from WSUCSH and 30/67 (44.8%) from Sodo Health Center.

3.4. Factors associated with maternal *Group B Streptococci* colonization

The assessment of the association of the socio-demographic, obstetric, and clinical characteristics with *GBS* colonization's is demonstrated in Table 3. During the study period, a total of 279 mothers were screened for *GBS* colonization; *GBS* was confirmed in 67 (24.0%) of the study participants. In this study, the highest

TABLE 1 Socio-demographic characteristics among pregnant women at selected health facility of Wolaita Sodo Town, Southern Ethiopia from June to August 2022 ($n = 279$).

Socio-demographic characteristics	Categories	Frequency	Percentage (%)
Health institutions			
WSU Comprehensive Specialized Hospital		169	60.6%
Sodo Health Center		110	39.4%
Age groups	15–19	22	7.9%
	20–24	53	19.0%
	25–29	135	48.4%
	30–34	48	17.2%
	≥ 35	21	7.5%
Residence	Urban	260	93.2%
	Rural	19	6.8%
Educational status	Primary	166	59.5%
	Secondary	55	19.7%
	College& above	58	20.8%
Marital status	Married	249	89.2%
	Divorced	6	2.2
	Single	21	7.5%
	Widowed	3	1.1
Monthly income	1,300–3,000	221 ≥ 10,000	79.2
	3,100–5,000	35	12.5
	5,100–1,000	20	20
	≥ 10,000	3	1.1

TABLE 2 Obstetric and clinical characteristics among pregnant women at a selected facility of Wolaita Sodo Town, Southern Ethiopia from June to August 2022 ($n = 279$).

Variables	Categories	Frequency	Percent (%)
Number of gravidities	Primigravida	173	62.0
	Multigravida	106	38
Gestational age (in weeks)	35	58	20.8
	36	146	52.3
	37	75	26.9
History of contraceptive usage	Yes	223	79.9
	No	56	20.1
History of abortion	Yes	51	18.3
	No	228	81.7
History of preterm labor	Yes	13	4.7
	No	266	95.3
History of Preterm prolonged rupture of membranes	Yes	–	–
	No	279	100
Diagnosis of UTI during pregnancy	Yes	33	11.8
	No	246	88.2
Diagnosis of STI during pregnancy	Yes	3	1.1
	No	276	98.9
History of ANC visit	Yes	279	100
	No	0	0
History of any antibiotic use	Yes	0	0
	No	279	100
History of any chronic medical illness	Yes	16	5.7
	No	263	94.3

PROM, prolonged rupture of membrane; ANC, antenatal care; CMI, chronic medical illness; UTI, urinary tract infection; STI, sexually transmitted infections.

prevalence of *GBS* was observed in those aged between 30 and 34 years 12/48 (25%), housewifes 32/120 (26.67%), and participants with a college or above education status 15/58 (25.9%). Based on history of contraceptive usage, higher rates of *GBS* were observed in those with a contraceptive usage history 53/223 (23.8%) compared with non-users. Out of 67 *GBS*-colonized pregnant women, 34 (19.7%) were Primigravida and 33 (31.1%) were multigravida. Of the mothers who were at 37 weeks of gestation, 25 (33.3%) were culture positive for *GBS*. Women with no history of abortion had a 24.1% rate of colonization and women with a recent history of abortion had a rate of 23.5%. Variable candidates for multivariate logistics regression were selected by considering $p < 0.25$ from the bivariate model. Multivariable logistic regression analysis showed that mothers whose educational status was at the college level and above had a significant association with an increased risk of *GBS* colonization ($p = 0.01$). In this study, educational status, gravidity, maternal age, and gestational age showed an association with *GBS* colonization in binary logistic regression but not in multi-logistic regression.

3.5. Antimicrobial susceptibility pattern of *GBS* isolates

The susceptibility patterns of *GBS* ($n = 67$) isolated from pregnant women were tested against nine antimicrobial agents (presented in Table 4). A high susceptibility rate of *GBS* isolate was seen for Penicillin G & Chloramphenicol (92.5%), Ampicillin and Ceftriaxone (89.6%), Vancomycin (74.62%), and Erythromycin (77%). Relatively, *GBS* showed high resistance to Tetracycline (88%), Ciprofloxacin (55.22%), and Clindamycin

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TABLE 3 Bivariate and multivariate analysis of socio-demographic and obstetric factors among pregnant women at selected health facilities of Wolaita Sodo Town, Southern Ethiopian from June to August 2022 ($n = 279$).

Characteristics		*GBS*		COR 95% CI	AOR 95% CI	*p*-value
Variables	Categories	Positive (%)	Negative (%)			
Age group	15–19	6 (27.3)	16 (72.7)	0.403 (0.089, 1.835)	0.380 (0.078, 1.861)	0.240
	20–24	13 (24.5)	40 (75.5)	0.543 (0.137, 2.153)		0.385
	25–29	32 (23.7)	103 (76.3)	0.9589 (0.258, 3.560)		0.949
	30–34	12 (25)	36 (67.6)	0.192 (0.050, 0.741)	0.300 (0.072, 1.254)	0.099
	>35	4 (19.0)	17 (81.0)	1	1	
Educational status	Primary	49 (29.5)	117 (70.5)	1	1	
	Secondary	3 (5.5)	52 (94.5)	0.812 (0.413, 1.596)		0.545
	College & above	15 (25.9)	43 (74.1)	6.047 (1.642, 22.370)	6.610 (1.724, 25.349)	0.01
Occupational status	Housewife	32 (26.7)	88 (73.3)	0.770 (0.376, 1.578)		0.475
	Civil servant	14 (21.9)	50 (78.1)	1.276 (0.501, 3.245)		0.609
	Student	10 (20.0)	40 (80.0)	0.770 (0.317, 1.870)		0.564
	Merchant	11 (24.4)	34 (75.6)	1	1	
Gravidity	Primigravida	34 (19.7)	139 (80.3)	1	1	
	Multigravida	33 (31.1)	73 (68.9)	0.499 (0.286, 0.871)	1.761 (0.941, 3.296)	0.077
Gestational age	35	14 (24.1)	44 (75.9)	1	1	
	36	28 (19.2)	118 (80.8)	1.433 (0.671, 3.061)		0.352
	37	25 (33.3)	50 (66.7)	2.204 (1.166, 4.164)	1.509 (0.700, 3.254)	0.15
Contraception	Yes	53 (23.8)	170 (76.2)	0.946 (0.474, 1.890)		0.875
	No	14 (25.0)	42 (75.0)	1	1	
Abortion	Yes	12 (23.5)	39 (76.5)	1.184 (0.569, 2.462)		0.651
	No	55 (24.1)	173 (75.9)	1		
Pretermlabor	Yes	5 (38.5)	8 (61.5)	0.698 (0.208, 2.345)		0.561
	No	62 (23.3)	204 (76.7)	1	1	
UTI	Yes	6 (18.7)	26 (81.3)			
	No	61 (24.7)	186 (75.3)	0.835 (0.345, 2.020)		0.689

UTI, unitary tract infection; COD, crude odds ratio; AOR, adjusted odds ratio.

(23.9%). Regarding the antibiogram of *GBS* isolates, susceptibility to all antibiotics was observed in two (2.98%) *GBS* isolate and resistance to one or more antibiotics was observed in 65 (97.01%) tested *GBS* isolates. According to the study, the most active drugs for *GBS* isolates were Penicillin, Chloramphenicol, Ampicillin, and Ceftriaxone, with susceptibility results of 92.5, 92.5, 89.6, and 74.62%, respectively. Moreover, five (7.46%) isolates of *GBS* showed intermediate susceptibility to Erythromycins and

TABLE 4 Antimicrobial susceptibility patterns of *GBS* isolated from pregnant women at selected health facility of Wolaita Sodo Town, Southern Ethiopia ($n = 67$).

Antibiotics with disc potency	Susceptible (%)	Intermediate (%)	Resistant (%)
Penicillin G (10 IU)	62 (92.5%)	–	5 (7.46%)
Ampicillin (10 μg)	60 (89.6%)	–	7 (10.4%)
Erythromycin (15 μg)	52/ (77%)	5/67 (7.46%)	10/ (14.92%)
Erythromycin (15 μg)	51/ (76.11%)	–	16/ (23.88%)
Vancomycin (30 μg)	50/ (74.62%)	6 (8.9%)	11 (16.41%)
Ceftriazone (30 μg)	60/ (89.6%)	–	7/ (10.4%)
Ciprofloxacin (5 μg)	30/ (44.77%)	–	37 (55.22%)
Chloramphenicol (30 μg)	62/ (92.5%)	5/ (7.5%)	–
Tetracycline (2 μg)	6/ (8.9%)	2/ (2.98%)	59/ (88%)

Chloramphenicol, six to Vancomycin (8.9%), and two (2.98%) to Tetracycline.

4. Discussion

The overall prevalence of *Group B Streptococcus (GBS)* in the present study among pregnant women was 24.0%. Such a result is comparable with studies worldwide, ranging from 10 to 30% in the USA, Kwatra et al. (2016) 6.5–36% in Europe (Nishihara et al., 2017), 7.1–16% in Asia (Centers for Disease Control and Prevention, 2007), and 11.9–31.6% in Africa (Arain et al., 2015). This study is also relatively similar to studies conducted in different parts of Ethiopia: 20.9% in Hawassa Health Centers (Mohammed et al., 2012), 19% in Jimma Hospital (Mengist et al., 2016), and 14.6% in different health centers in Addis Ababa (Assefa et al., 2018). The rate of *GBS* colonization in this study is lower than the studies conducted in Brazil at 28.4% (Melo et al., 2018) and South Africa at 30.9% (Bolukaoto et al., 2015).

The rate of *GBS* found in this study and some countries of Europe is comparable, for example in Italy two studies reported *GBS* rates as 17.9% (Melo et al., 2018) and 18% (Poyart et al., 2003). In Switzerland and Poland, positivity rates were 21% (Cockerill et al., 2012) and 17.2% (Blumberg et al., 1996) respectively. A study done in the Netherlands showed 21% (Flaherty et al., 2019). However, a lower *GBS* colonization rate was recorded from Istanbul and Elazin in Turkey giving 8% (Khan et al., 2015) and 8.7% (Assefa et al., 2018), respectively, while a study in Northern Greece reported the lowest rate of 6.6% (Valkenburg-Van Den Berg et al., 2006).

GBS colonization is an important cause of infection in pregnant women and is associated with adverse outcomes in their newborns; however, there have been limited studies available in Ethiopia (Assefa et al., 2018). It also has variable prevalence and susceptibility against commonly prescribed drugs in different geographic locations.

Providing adequate knowledge for pregnant women on *GBS* risk factors plays a crucial role in decreasing the morbidity and mortality related to maternal *GBS* infections. The geographical differences, variability in the sample size, and methods employed for *GBS* detection might possibly explain the disparities.

In this study, socio-demographic characteristics (age, residence, education status, marital status, income, and occupation) and Obstetrics and clinical characteristics (gravidity, gestational age, history of Preterm PROM, preterm labor, contraceptive use, history of abortion, UTI pregnancy, STI pregnancy, and any antibiotic) had no relation to the *GBS* colonization. Similar findings were reported from studies done in Italy that reported the *GBS* rate as 17.9% (Melo et al., 2018), Poland had a positivity rate of 17.2% (Blumberg et al., 1996). Having a college or above educational level was, however, significantly associated with maternal colonization ($p = 0.01$), with a similar finding shown in studies done in Poland (El Aila et al., 2010) and Bangladesh (Blumberg et al., 1996). Maternal age and gestational age were identified as risk factors for *GBS* colonization (Blumberg et al., 1996; Poyart et al., 2003; El Aila et al., 2010; Melo et al., 2018) in studies done before but no association was seen in the current study. In study done in Thailand reported that lower maternal age and lower gestational age were risks for colonization by *GBS* (Khan et al., 2015). The relationship between these factors and *GBS* colonization, however, showed marked inconsistencies.

In some studies, colonization increased with age (Cockerill et al., 2012), while other reports confirmed younger age groups showed the highest risk (Khan et al., 2015; Assefa et al., 2018). The possible reason for this difference seems to be seasonal differences globally, the availability of laboratory facilities for detecting *GBS*, and the length of study periods.

In this study, maternal age, gravidity, and gestational age showed association with *GBS* colonization in binary logistic regression, and college and above educational level showed an association on multi-logistic regression.

In the current study, the susceptibility pattern of *GBS* isolates to Penicillin (92.5%), Vancomycin (74.62%), ampicillin (89.6%), Ceftriaxone (89.6%), Chloramphenicol (92.5.%), Erythromycin (77%), and Clindamycin (76.11%) is comparable with previous studies conducted in different countries, in which similar records were found from Mekele, Ethiopia (Alemseged et al., 2015), the USA (Rausch et al., 2009), Canada (Ayata et al., 1994), and Lebanon (El-Kersh et al., 2002).

However, high resistance was observed in Tetracycline (88%), Ciprofloxacin (55.22%), and Clindamycin (23.88%). Similar records were found in Lebanon (El-Kersh et al., 2002), the USA (Rausch et al., 2009; Strus et al., 2009), Mekele, Ethiopia (Alemseged et al., 2015), and Canada (Ayata et al., 1994). Erythromycin (14.92%), Vancomycin (16.41%), Ceftriaxone and Ampicillin (10.4%), and, *GBS* resistant with reduced Penicillin susceptibility have been detected. Penicillin is the first agent for the prevention and treatment of GBS infections; however, nowadays GBS strains with reduced susceptibility to Penicillin have been reported periodically as seen in different study previously like also this study.

To prevent *GBS*, Erythromycin and Clindamycin are alternative antibiotics to Penicillin, especially for pregnant women with allergies who are at a high risk of anaphylaxis.

The increase of *GBS* strains resistant to Erythromycin and Clindamycin is complicating the management of pregnant women

who are allergic to Penicillin (Valkenburg-Van Den Berg et al., 2006). In contrast to reports from many other countries, the highest susceptibility in the present study was seen to Erythromycin (77%) and Clindamycin (76.11%), and only a few isolates were resistant to these drugs. In this study, 15% Erythromycin and 24% Clindamycin resistance was reported, which is similar to studies done in Ethiopia, Gondar, showing a 22.7% resistance to Erythromycin and 17.6–18.2% resistance to Clindamycin (Schmidt et al., 1989). Also in South Africa, 17.2% Clindamycin and 21.1% Erythromycin resistance were reported (Verani et al., 2010). In Italy and the USA, Clindamycin resistance was reported at 17.6% (Savoia et al., 2008) and 21.0% (Fatemi et al., 2009), respectively, which is comparable to our study.

Generally, in contrast to this study, worldwide studies have reported a high resistance rate to Erythromycin, which ranges from 18 to 54% (Blumberg et al., 1996). Absence or low antibiotic resistance of *GBS* strains in the present study may indicate the suitability of Penicillin, Ampicillin, and Chloramphenicol for Ethiopia to prevent *GBS* until a vaccine is available on the market.

Similar to the present study, low levels of resistance to Erythromycin was reported in Australia (6.4%) (Assefa et al., 2018), Brazil (4.1%) (De Steenwinkel et al., 2008), on the Thai-Myanmar border (8.5%) (El Aila et al., 2010), and France (4%) (Blumberg et al., 1996). No resistance to Chloramphenicol was observed in this study and two (7.5%) and three (10.4%) of the isolates showed resistance to Penicillin and Ampicillin, respectively.

High resistance to Tetracycline (88%) in this study was reported and similar reports from other countries, such as Brazil (83%) (Valkenburg-Van Den Berg et al., 2006), Australia (85.9%) (Savoia et al., 2008), Kuwait (89.5%) (Rausch et al., 2009), Canada (89%) (Ayata et al., 1994), and Greece (85%) (Tsolia et al., 2003) also reported.

CDC-approved patients can take Penicillin or Ampicillin if they are not allergic to Penicillin. Clindamycin or Vancomycin is the drug of choice for those who have a major Penicillin allergy and Ceftriaxone for a minor allergy to Penicillin (YektaKooshali et al., 2018). It is difficult to develop a vaccine for *GBS* due to its multiple serotypes and variations in geographical locations (Garland et al., 2000). In addition, resistance to Clindamycin and Erythromycin, which are first-line drugs for those with a penicillin allergy, has increased rapidly (Garland et al., 2000).

Resistance to Erythromycin ranged from 7 to 40% and Clindamycin from 3 to 26.4%; this was related to some serotypes (Fatemi et al., 2009; Tsui et al., 2009; Khan et al., 2015). Inappropriate use of antimicrobial drugs leads to high drug resistance. In Ethiopia, people can easily go to pharmacies without a prescription to buy antibiotics and this type of antibiotic use might responsible for the high drug resistance rates observed currently.

4.1. The strengths of this study

In this study, a more valid method was used to identify *GBS* colonization. The use of THB (Todd-Hewitt broth), a primary selective broth media for isolation of *GBS* that consisted of 10 μg/mL colistin and 15 μg/mL nalidixic, as well as the use of antibiotics in the primary media to selectively isolate the bacteria, makes our isolation able to indicate maximum carriage rate.

4.2. Limitations of the study

- No serotyping was done and only disc diffusion was used for the antibiotic susceptibility test.
- There was a failure to assess the outcome on neonates whose mothers were detected to be colonized by *GBS* in the study.

5. Conclusion

The prevalence of *GBS* in the current study was 24.0%. Among 279 pregnant women, the carriage rate of *GBS* was highest among those aged 30–34 years. Having a college or above level of maternal education was significantly associated to maternal colonization in the current study [AOR = 6.610, 95% CI (1.724–25.349), $p = 0.01$]. The highest susceptibility was shown for penicillin and chloramphenicol (92.5%). High resistance was observed against Tetracycline (88%) and Ciprofloxacin (55.22%). The overall prevalence of *GBS* in the current study is high. Therefore, there is a need for screening of pregnant mothers near term delivery and to determine their antibiotic susceptibility so as to set appropriate intervention mechanisms. Early diagnosis, treatment, and proper management is very important to reduce *GBS* infection of neonates and newborns.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Institutional Review Board of College of Health Sciences and Medicine, Wolaita Sodo University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – review & editing. BG: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft. SS: Conceptualization, Data curation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing. WW: Conceptualization, Software, Supervision, Validation, Writing – review & editing. TS: Data curation, Formal analysis, Software, Supervision, Validation, 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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The analysis of *Lactobacillus* spp. distribution in the vaginal microbiota of Polish women with abnormal Pap smear result

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Introduction: A healthy vaginal microbiota is represented mainly by *Lactobacillus* spp. and plays a vital role in maintaining the functional balance in the vaginal environment. Scientists have drawn attention to possible correlations between the vaginal microbiome and gynecological neoplasms. Several recent studies have shown a potential link between the vaginal microbiome and the risk of developing cervical cancer from human papillomavirus (HPV) infection. This study aimed to compare the prevalence and abundance of various lactic acid bacteria species (LABs) in vaginal swabs from healthy controls and patients with abnormal Pap smear results.

Methods: The study included 100 women (79 patients with abnormal cervical Pap smear results and 21 controls) from whom vaginal swabs were collected. Real-time quantitative PCR was used to determine seven lactic acid bacteria (LAB) species and their quantities.

Results: Most patients were colonized by two *Lactobacillus* species, primarily *Lactobacillus gasseri* (93%) and *L. crispatus* (83%). Patient age and place of residence were associated with the diversity of LAB in the vaginal microbiota. The abundance of *L. delbrueckii* in the vaginal microbiota increased, whereas the abundance of *L. gasseri* abundance decreased, with patient age. *Lactobacillus acidophilus* and *Limosilactobacillus fermentum* were significantly more often detected in patients living in rural versus urban areas. Statistical analysis did not show any significant differences in LAB between groups of patients with various changes on smear tests.

Discussion: The degree of dysplastic changes in the endothelium or the presence of a group of atypical cervical stratified epithelial cells was not associated with significant changes in the studied vaginal bacteria.

KEYWORDS

vaginal microbiota, *Lactobacillus*, *L. gasseri*, *L. crispatus*, HPV, cervical cancer

1. Introduction

Bacteria, viruses, archaea, yeast, and fungi form the human microbiome (Marchesi and Ravel, 2015; Sender et al., 2016; Requena and Velasco, 2021), which colonizes the skin, genitourinary system, oral cavity, and intestinal tract. The human microbiota establishes a symbiotic relationship with the host and plays a vital role in maintaining the host's physiological homeostasis (Dominguez-Bello et al., 2019). The composition of the microbiota differs depending on the site of microbial colonization. A healthy vaginal microbiota is represented mainly by *Lactobacillus* spp. including *L. crispatus*, *L. jensenii, L. gasseri,* and *L. iners* (Requena and Velasco, 2021), whereas *Prevotella*, *Gardnerella*, *Atopobium*, *Sneathia*, *Bifidobacterium*, *Megasphaera*, and *Anaerococcus* are associated with vaginosis (Moreno and Simon, 2018). The microbiota plays a vital role in maintaining functional balance in the vaginal environment and in preventing colonization by pathogens and the overgrowth of commensal microorganisms. *Lactobacillus* spp. stabilize the vaginal microbiota by producing antimicrobial components such as hydrogen peroxide, lactic acid, and bacteriocin-like compounds. The ability to adhere and compete with other harmful microorganisms is also crucial (Borges et al., 2014; Tachedjian et al., 2017). In most areas of the human body, a highly species-diverse microbiome is a sign of health (Turnbaugh et al., 2007; Flores et al., 2014). However, for the female reproductive system, a healthy state is more often associated with low microbial diversity and the dominance of one or more *Lactobacillus* species (Ravel et al., 2011; Liu et al., 2013; MacIntyre et al., 2015). An increasing number of publications indicate a correlation between disturbances of the vaginal microbiota, bacterial vaginosis, and the development of fungal, bacterial, and viral infections (Marchesi and Ravel, 2015; Mitra et al., 2016; Sender et al., 2016; Requena and Velasco, 2021). Some studies have demonstrated a correlation between bacterial vaginosis and increased persistence of human papillomavirus (HPV) infections as well as the development of cervical intraepithelial neoplasia (Gillet et al., 2011; King et al., 2011; Gillet et al., 2012; Guo et al., 2012; Champer et al., 2018). Women with persistent HPV infections have a more species-diverse microbiome, rich in *L. gasseri* and *Gardnerella vaginalis* and at the same time with a low abundance of *Lactobacilli* (Gao et al., 2013; Lee et al., 2013). The rate of resolution of HPV infection and development cervical cancer might also depend on the composition of the vaginal microbiome (Brotman et al., 2014). Apart from vaginal microbiota, also cervical microbiome is important for female's health. Higher cervical species diversity has been demonstrated in severer cervical intraepithelial neoplasia of reproductive-age women (Wu et al., 2021). More complex composition of cervical microbiota which tends to progress with the aggravation of lesions as well as the prevalence of anaerobic bacteria were found in women with cervical pathology. Disturbed cervical microbial community is associated with cervical intraepithelial neoplasia and cervical cancer (Wang et al., 2022). Lower relative abundance of *Lactobacillus* as well as presence of *Porphyromonas*, *Prevotella* and *Campylobacter* and *Sneathia* have been suggested to promote the development of cervical pathology.

Cervical cancer is the fourth most common cancer in the world in terms of both incidence and mortality. In 2018, nearly 570,000 new patients were diagnosed globally, and 311,000 women died from cervical cancer (Arbyn et al., 2020). This cancer can be prevented through early diagnosis and treatment of precancerous states, but it remains the most common cancer in most low and middle-income countries (Arbyn et al., 2020). This study aimed to compare the distribution of 7 various vaginal *Lactobacillus* spp. between Polish women with abnormal Pap smear results (study group) and healthy individuals (control group) based on quantitative analysis of bacteria in vaginal swabs. We also assessed correlations between the vaginal microbiota and patient age, place of residence (urban vs. rural), HPV status, menopausal status, and history of vaginal infection within the past 3 months.

2. Materials and methods

2.1. Participants

The study included 100 patients (aged 18–72 years) from Ist Department of Gynecological Oncology and Gynecology, Medical University in Lublin, Poland. Inclusion criteria were admission to Gynecology Department and expressed willingness to participate in the project. Exclusion criteria were lack of consent, undergoing surgical procedures in the lower genital area, age below 18, inability to collect accurate data for the project. Patients with abnormal Pap smear results were included in the study group, and healthy individuals were used as a control group. TBS system, which provides a uniform format and standardized lexicon for cervical/vaginal cytology reports was used to classify abnormal Pap smears (Chatterjee et al., 2000). Vaginal swabs were collected from all study participants from October 2014 to November 2017. Considering the size of women population aged 18–72 years in Poland is approximately 9.5 mLn, the margin of error of the measured value at the 95% confidence interval is ±6% when tested 100 women.

This study was approved by the Bioethics Committee of Medical University in Lublin KE – 0254/174/2014 and was conducted in accordance with the fundamental ethical principles described in the Declaration of Helsinki (World Medical Association, 2013). All participants signed informed consent to participate in this study.

2.2. Vaginal swabs for genetic testing

Vaginal swabs were collected from patients using sterile silicone brushes (Genomic Micro AX Swab Gravity Plus kit, A&A Biotechnology). Swabs were placed in 1.5 ml tubes with 700 µL Spheroid Lysis Buffer (SLB) (A&A Biotechnology). All specimens were stored in ultralow freezer at -80°C until further analyses. DNA isolation was performed within 6 months from the date of swab collection. The extraction resulted in high-quality DNA that is free of inhibitors for reproducible results.

2.3. Real-time quantitative PCR

Real-time quantitative PCR was performed on 100 samples of DNA isolated from vaginal swabs from 79 patients with abnormalities in the smear test (50 patients with low-grade and 29 patients with high-grade epithelial lesions) and 21 healthy patients (control group) using LightCycler 96 (Roche). The quantitative analysis included

TABLE 1 Sequences of primers used in real-time PCR reactions.

	Primer F	Primer R	Reference
L. acidophilus	GGRTGATTTGTTGGACGCTAG	GCCGCCTTTCAAACTTGAATC	Haarman and Knol (2006)
L. crispatus	TGGAAACAGRTGCTAATACCG	CAGTTACTACCTCTATCTTTCTTCACTAC	Byun et al. (2004)
L. delbrueckii	TGGATCACCTCCTTTCTAAGGAAT	TGTTCTCGGTTTCATTATGAAAAAATA	Zhang et al. (2012)
L. fermentum	TGCTTGCATCTTGATTTAATTTTG	GGTTCTTGGATYTATGCGGTATTAG	Zhang et al. (2012)
L. gasseri	GAAAGAGCCCAAACCAAGTGATT	CTTCCCAGATAATTCAACTATCGCTTA	Byun et al. (2004)
L. plantarum	AGCGAGCGGAACTAACAGATTTAC	AGCTGATCATGCGATCTGCTT	Haarman and Knol (2006)
L. rhamnosus	GCACCTGATTGATTTTGGTCG	GTCCATTGTGGAAGATTCCC	Zhang et al. (2012)

TABLE 2 Demographic and clinical data of the enrolled participants.

Parameters	*N* (%)
Place of residence	
City	33
Village	67
Menopausal status	
Postmenopausal	18
Premenopausal	82
TBS diagnosis	
Normal	21
Atypical squamous cells of undetermined significance	24
Atypical squamous cells, cannot exclude a high-grade squamous intraepithelial lesion	11
Low-grade squamous intraepithelial lesion	26
High-grade squamous intraepithelial lesion	18
Human papillomavirus diagnosis	
Positive	23
Negative	23
Unknown	54
Vulvovaginitis within the last 3 months	
Occurred	28
Not occurred	72
Vaccination against human papillomavirus	
Yes	0
No	100

TBS, The Bethesda System for Reporting Cervical Cytology.

seven *Lactobacillus* species (*Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus delbrueckii*, *Limosilactobacillus fermentum*, *Lactobacillus gasseri*, *Lactiplantibacillus plantarum*, and *Lacticaseibacillus rhamnosus*). The quality control (QC) of the implementation procedure included IC (internal control) with amplification for the IC within normal limits, HC (high copy) control, LC (low copy) control, a normal control (NC) with amplification for IPC, and NTC (no template control) with no amplification. Standards, positive controls, and negative controls were performed in triplicate for each analysis. The primers (Genomed, Warsaw, Poland) presented in Table 1 were used for the detection and quantification of the seven LAB species.

2.4. Statistical analysis

The Shapiro–Wilk test was used to check the normality of the data distribution. The verification of statistical hypotheses was based on non-parametric tests. The non-parametric Mann–Whitney test was used to compare the two groups (study and control). The non-parametric Kruskal-Wallis test was used to compare more than two groups (if this test showed a statistically significant difference between the groups, the post-hoc test was used for further comparisons). Spearman's rank correlation coefficients were calculated to analyze the coexistence of various bacterial species. The Pearson Chi-square test and Fisher's exact test were used to evaluate the relationship between two qualitative features. STATISTICA 13.1 was used to perform statistical analyses. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Clinical background of the study subjects

In our study, most females were premenopausal. Over 50% of study participants have not been tested for HPV infection and none of them have been vaccinated against it. Vulvovaginitis within lat 3 months was reported in 28% of enrolled females. The results of smear test were normal in 21% of participants, ASC-US was found in 24%, ASC-H in 11%, LSIL in 26% and HSIL in 18% of females. The demographic and clinical data of participants are summarized in Table 2. The vaginal microbiota of most participants (55%) was dominated by two of the seven LABs investigated. Three species were observed in 17% of the examined participants, four in 11%, and five in 3% of participants. In 2% of patients, none of the studied LABs was found. Two LABs tested were detected in 53.16% of the vaginal swabs collected from patients with abnormal Pap smear results (study group) and in 61.91% of individuals from the control group (Figure 1).

3.2. Associations between clinical findings and *Lactobacilli*

There were no statistically significant differences in identified LAB species between groups of patients with normal Pap smear results and those with ASC-US, ASC-H, LSIL, and HSIL ($p > 0.05$). In general, *L. gasseri* and *L. crispatus* were the most common LAB

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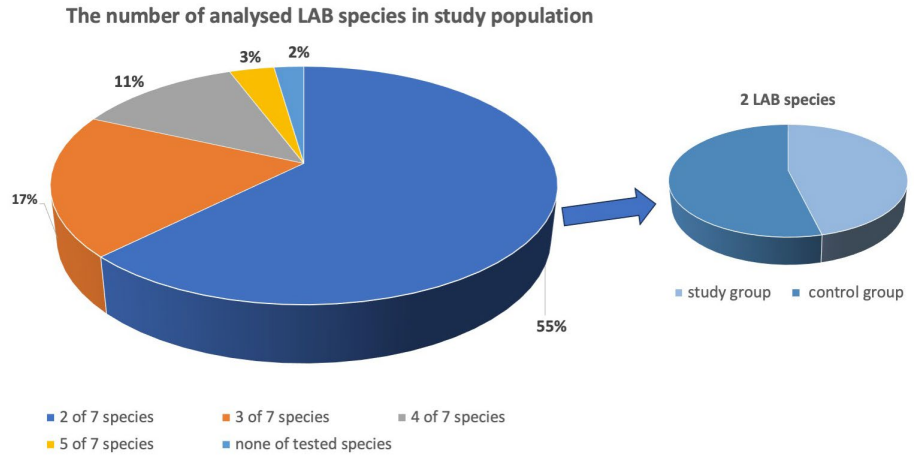

FIGURE 1
Number of analyzed LAB species in study participants.

TABLE 3 Prevalence of LAB species detected in enrolled participants in relation to cervical Pap smear result.

	Total *n* = 100	Negative Pap smear (normal) *N* = 21	LSIL *N* = 26	HSIL *N* = 18	ASC-US *N* = 24	ASC-H *N* = 11	*p* value
N (%)							
L. acidophilus	2 (2.0)	4 (4.8)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	0.52
L. crispatus	83 (83.0)	17 (81.0)	23 (88.5)	16 (88.9)	18 (75.0)	9 (81.2)	0.71
L. delbrueckii	25 (25.0)	6 (28.6)	7 (26.9)	6 (33.3)	4 (16.7)	2 (18.2)	0.72
L. fermentum	9 (9.0)	2 (9.5)	3 (11.5)	2 (11.1)	2 (8.3)	0 (0.0)	0.67
L. gasseri	93 (93.0)	20 (95.2)	24 (92.3)	16 (88.9)	16 (91.7)	11 (100)	0.69
L. plantarum	4 (4.0)	1 (4.8)	2 (7.7)	0 (0.0)	1 (4.2)	0 (0.0)	0.54
L. rhamnosus	15 (15.0)	5 (23.8)	4 (15.4)	1 (5.6)	4 (16.7)	1 (9.1)	0.54

LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; ASC-H, atypical squamous cells, cannot exclude a high-grade squamous intraepithelial lesion.

species detected in all enrolled patients (Table 3). *L. gasseri* and *L. crispatus* were also most detected in patients with ASC-US, ASC-H, LSIL, and HSIL. The occurrence of LAB species in participants with various Pap smear results is summarized in Table 3.

The quantitative real-time PCR analysis also demonstrated the numbers of *L. crispatus* and *L. gasseri* cells were the highest. Statistical analysis did not show any significant differences in the number of cells of each species between the studied subgroups of patients (Figure 2). What's interesting, *L. acidophilus* was observed only in patients with HSIL and normal smear, while *L. plantarum* in women with ASC-US, LSIL and normal smear.

Moreover, analysis of the species distribution in different age groups showed that the abundance of *L. delbrueckii* significantly increased, whereas that of *L. gasseri* decreased, with age (Table 4). Statistical analysis of *L. gasseri* in three age groups also showed a statistically significantly higher abundance of this bacterium in swabs taken from patients aged 18–29 years ($p = 0.0046$). *L. delbrueckii* was significantly more abundant in swabs obtained from the oldest group of patients (47–72 years) ($p = 0.057$).

Lactobacillus acidophilus (6.1%) and *L. fermentum* (18.2%) were found significantly more frequent among women living in rural areas than among those living in urban areas (*L. acidophilus* 0%, *L. fermentum* 4.5%) (Table 5). Also, the abundance of *L. acidophilus* cells (range 0–5.7×10^6 vs. 0, $p = 0.044$) and *L. fermentum* cells (range 0–7.2×10^7 vs. 0) was higher in women living in rural areas than in those living in urban areas (0–1.6×10^6, $p = 0.029$).

There were no statistically significant differences in the vaginal microbiota in patients HPV positive (23 patients) and without HPV infection (23 patients), in those with and without vaginitis within the last 3 months, and between premenopausal and post-menopausal participants (Table 6).

3.3. Correlations between different *Lactobacilli* species

In patients with abnormal cervical Pap smear result significant positive correlations were identified for *L. delbrueckii* and *L. gasseri*

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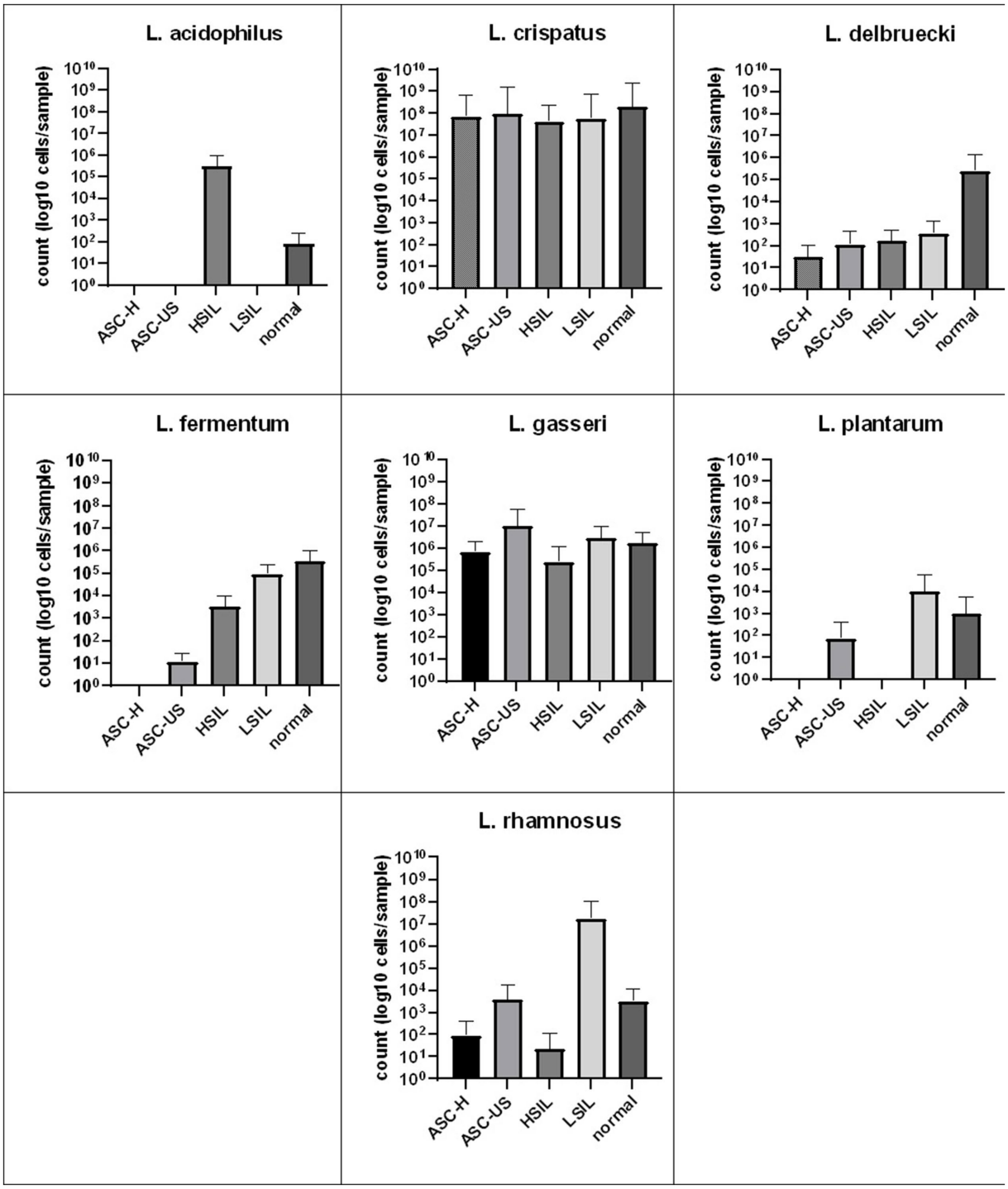

FIGURE 2
Comparison of the average number of LAB bacterial cells detected by real-time PCR from vaginal swabs in relation to cervical Pap smear result. SD, standard deviation; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; ASC-H, atypical squamous cells, cannot exclude a high-grade squamous intraepithelial lesion.

($r = 0.79$, $p = 0.048$), and negative correlation between *L. fermentum* and *L. plantarum* ($r = -0.79$, $p = 0.048$). In healthy control group, significant correlation between *L. delbrueckii* and *L. rhamnosus* ($r = 0.59$, $p = 0.005$) and *L. pantarum* and *L. fermentum* ($r = 0.72$, $p = 0.0002$) was detected indicating the coexistence of these species (Figure 3).

4. Discussion

The vaginal microbiome plays a vital role in keeping the female reproductive system healthy. Bacterial vaginosis is a state in which the natural balance of bacteria in the vagina is disturbed, which has been linked to a wide variety of health problems, including a higher risk of

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TABLE 4 Analysis of LAB species distribution according to participant age.

	Age groups, *n* (%)			*p* value
	18–29 (*n* = 26)	30–46 (*n* = 45)	47–72 (*n* = 29)	
L. acidophilus	0	2 (4.4)	0	0.29
L. crispatus	20 (76.9)	38 (84.4)	25 (86.2)	0.62
L. delbrueckii	2 (7.7)	12 (26.7)	11 (37.9)	0.06
L. fermentum	2 (7.7)	4 (8.9)	3 (10.3)	0.94
L. gasseri	26 (100)	43 (95.6)	24 (82.8)	0.005
L. plantarum	0	3 (6.7)	1 (3.45)	0.38
L. rhamnosus	1 (3.85)	8 (17.8)	6 (20.7)	0.17

TABLE 5 LAB species distribution according to participant place of residence.

	Number of patients (%)		*p* value
	Rural (*n* = 33)	Urban (*n* = 67)	
L. acidophilus	2 (6.1)	0	**0.04**
L. crispatus	30 (90.1)	53 (79.1)	0.14
L. delbrueckii	7 (21.2)	18 (26.9)	0.54
L. fermentum	6 (18.2)	3 (4.5)	**0.03**
L. gasseri	31 (93.4)	62 (92.5)	0.80
L. plantarum	3 (9.1)	1 (1.5)	0.068
L. rhamnosus	6 (18.2)	9 (13.4)	0.53

Bold values mean statistical significance.

sexually transmitted diseases, cystitis, postoperative complications, infertility, miscarriages, premature births, intrauterine infections, cervical and uterus infections, dysplasia, and cervical cancer. Also, cervical microbiota is crucial since the cervix is the place of infections caused by pathogens such as human immunodeficiency virus (HIV) and HPV, *Chlamydia trachomatis*, or *Neisseria gonorrhoeae*.

In our present study, vaginal microbiota of most participants in both groups was dominated by two of the seven LABs investigated. Three species were observed in 17% of the examined participants, four in 11%, and five in 3% of participants. This study revealed the presence of LAB belonging to seven species (*L. acidophilus*, *L. crispatus*, *L. delbrueckii*, *L. fermentum*., *L. gasseri*, *L. plantarum*, and *L. rhamnosus*) in the material from vaginal swabs, with *L. gasseri* being most frequently detected, and *L. crispatus being most abundant one.* The dominance of *Lactobacillus crispatus* and *L. gasseri* is observed in community state types (CST) I and II group, respectively (Ravel et al., 2011). The presence of *L. gasseri* was suggested to be associated with rapid clearance of acute HPV infection (Brotman et al., 2014). It has to be noted that HPV infection itself was demonstrated to modify the mucosal metabolism and host immunity, thus promoting changes in the vaginal microbiota (Scott et al., 1999). The results of studies indicate that *Lactobacillus* species are capable of adhering tightly to the vaginal epithelium, covering its surface, thus protecting the vagina from colonization by pathogenic microorganisms (Petricevic et al., 2014; Mitra et al., 2016). The presence of microbiome dominated by *Lactobacilli* is related with well-balanced immune-tolerant vaginal microenvironment (Donnarumma et al., 2014). However, it was observed that not all the species equally contributed.

Lactobacillus gasseri was the most frequently detected species in our study, whereas *L. crispatus* was the most abundant one. Similar results were obtained by De Backer et al. (2007), who examined 71 vaginal swabs collected from Belgian women. They demonstrated that, among four *Lactobacillus* species (*L. crispatus*, *L. iners*, *L. jensenii*, and *L. gasseri*), *L. crispatus* was the most common (detected in 93% of the samples); *L. iners* was detected in 75% of swabs, *L. gasseri* in 73%, and *L. jensenii* in 46%. In another study, *L. crispatus* was the most abundant *Lactobacillus* species in the samples collected from healthy women and from women with bacterial vaginosis (Zozaya-Hinchliffe et al., 2010). In the vaginal and cervical swabs of 96 women from Florida, *L. iners* (55%), *L. crispatus* (29%), *L. gasseri* (13%), and *L. jensenii* (13%) were the most common species. In another American study, *L. iners* was the most abundant species in healthy women and in those with bacterial vaginosis (Pavlova et al., 2002). A meta-analysis by Norenhag et al. (2020) showed that *Lactobacillus* in the vaginal microbiome of 55% of HPV-positive and 38% of HPV-negative women was sparse. However, in 44% of HPV-positive and 58% of HPV-negative women, the vaginal microbiome was rich in *L. crispatus* and *L. iners*.

In this study, we failed to observe statistically significant differences between groups of patients with normal Pap smear results and those with ASC-US, ASC-H, LSIL, and HSIL. The number of cells of a given bacteria appears to be of higher importance than the presence itself. In our study, quantitative analysis demonstrated the highest abundance of *L. crispatus* and *L. gasseri* cells. However, again we failed to show any significant differences in cells number of each species between the studied subgroups of patients. No statistically significant differences in the vaginal microbiota in HPV positive and HPV negative patients, in those with and without vaginitis within the last 3 months, and between premenopausal and post-menopausal participants were observed. In contrast, a study among African women showed that a cervical microbiota dominated by *Lactobacillus* spp. (*L. crispatus, L. inners*) was associated with a lower incidence of HIV, herpes simplex virus 2 (HSV-2), and high-risk HPV strains (Borgdorff et al., 2014). The abundance of *L. gasseri* and *L. iners* in the vagina has been associated with the rapid resolution of HPV infection. By contrast, low levels of *Lactobacillus* spp. and high levels of *Atopobium* spp. have been associated with slower resolution of HPV infection (Gao et al., 2013; Lee et al., 2013; Brotman et al., 2014). Vaginal infections with *C. trachomatis* have also been suggested to enhance women's susceptibility to HPV infections via altering the composition of the vaginal microbiota (Silva et al., 2014). Several studies have focused on the correlation between the vaginal microbiome and cervical dysplasia. Overall, a vaginal microbiome rich in *L. crispatus* occurs in a healthy state (Oh et al., 2015; Audirac-Chalifour et al., 2016; Seo et al., 2016), whereas *L. iners* seems to be related to cervical cancer either alone or associated with HPV infection (Oh et al., 2015; Piyathilake et al., 2016; Seo et al., 2016). However, another study showed the opposite relationship, with *L. iners* associated with a reduced risk of cervical intraepithelial neoplasia and cervical cancer (Audirac-Chalifour et al., 2016). Other *Lactobacillus* species have also shown differing associations with cervical dysplasia (Mitra et al., 2015; Piyathilake et al., 2016).

In this study, we observed a strong positive correlation between *L. delbrueckii* and *L. gasseri* as well as negative correlation between

TABLE 6 Occurrence of LAB species in patients by HPV, vaginitis, and menopausal status.

	HPV-positive	HPV-negative	*p* value	Vaginitis-positive	Vaginitis-negative	*p* value	Pre menopausal	Post menopausal	*p* value
	N = 23	*N* = 23		*N* = 28	*N* = 72		*N* = 82	*N* = 18	
L. acidophilus	0 (0)	0	1.0	1 (3.6)	1 (1.4)	0.48	2 (2.4)	0 (0)	1.0
L. crispatus	21 (91.3)	17 (73.9)	0.24	20 (71.4)	63 (87.5)	0.075	68 (82.9)	15 (83.3)	1.0
L. delbrueckii	5 (21.7)	3 (13.0)	0.70	8 (28.6)	17 (23.6)	0.61	18 (21.9)	7 (38.9)	0.14
L. fermentum	2 (8.7)	3 (13.0)	1.0	2 (7.1)	7 (9.7)	1.0	7 (8.5)	2 (11.1)	0.66
L. gasseri	22 (95.7)	20 (87.0)	0.61	26 (92.9)	67 (93.1)	1.0	77 (93.9)	16 (88.9)	0.61
L. plantarum	0 (0)	1 (4.4)	1.0	2 (7.1)	2 (2.8)	0.31	4 (4.9)	0 (0)	1.0
L. rhamnosus	3 (13.0)	4 (17.4)	1.0	4 (14.3)	11 (15.3)	1.0	11 (13.4)	4 (22.2)	0.46

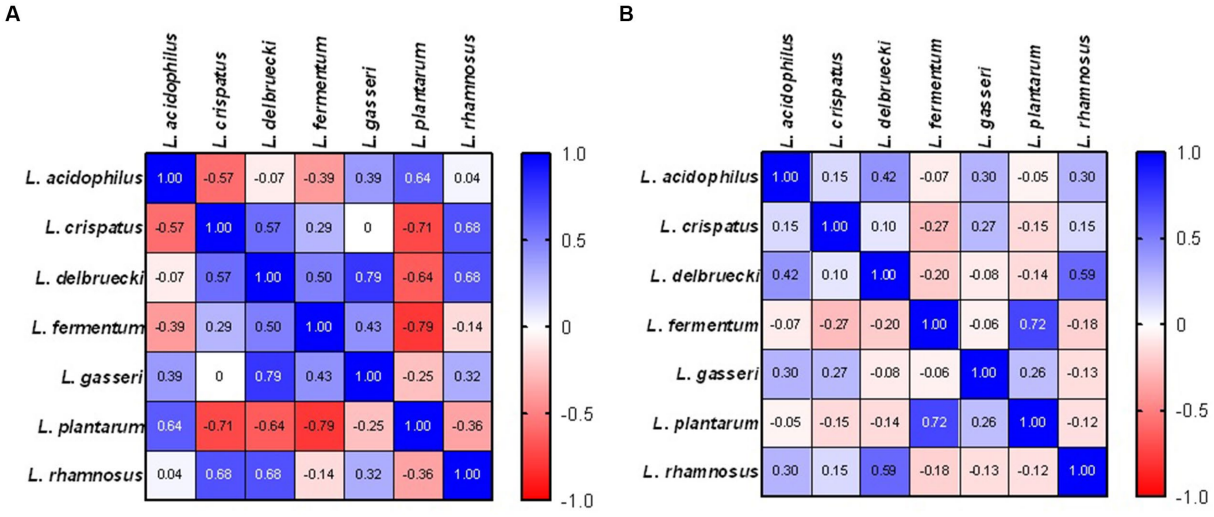

FIGURE 3
Correlations indicating the co-occurrence of individual LAB species in patients with abnormal cervical Pap smear result **(A)** and healthy control **(B)**.

L. fermentum and *L. plantarum* in patients with abnormal cervical Pap smear. In turn, in healthy control group, the presence of *L. delbrueckii* correlated with *L. rhamnosus* as well as between *L. pantarum* and *L. fermentum*. We did not observe correlation between *L. crispatus* and any other species. Similary, Gajer et al. (2012) reported that this bacteria is rarely coexisting with other bacterial species since it is either strongly dominant, or absent. Moreover, it was suggested that *L. crispatus* is associated with protection from pathogens and is the least likely to transition into CST IV. Women with such microbiota are less likely to become infected with Herpes simplex virus (HSV)-2 and HIV, as well HPV (Borgdorff et al., 2014). Vaginal microbial communities exist in a state of dynamic equilibrium which provides resilience (Ravel et al., 2011). The coexistence of various *Lactobacillus* species in the vaginal milieu enables the maintenance of homeostatic stage. Bacteria show different pH preference, growth patterns and lactic acid production capability (Mehta et al., 2020). The coexistence preferences have been shown to play a key role in the modulation of bacterial taxa composition in the vaginal ecosystem (Verstraelen et al., 2009).

In our study, *L. acidophilus* was observed only in patients with HSIL and normal smear, while *L. plantarum* in women with ASC-US, LSIL and normal smear. Similarly, Kwasniewski et al. (2018) observed the prevalence of *Lactobacillus acidophilus,* but also *Lactobacillus iners* and *Lactobacillus crispatus* in women diagnosed with HSIL. Moreover, in their study *Lactobacillus acidophilus* and *Lactobacillus iners* were predominant in CST diagnosed with LSIL HPV(+), while *Lactobacillus crispatus* was absent in smears.

Moreover, in our study, the analysis of the species distribution in different age groups showed that the abundance of *L. delbrueckii* significantly increased, whereas that of *L. gasseri* decreased, with age. *L. acidophilus* and *L. fermentum* were significantly more prevalent and the number of their cells were much higher among women living in rural areas compared with those living in urban areas.

Apart from the area of residence, the composition of the vaginal microbiota differs between various races. The composition of vaginal microbiome differs between women in different geographic regions therefore, the comparison of results obtained in studies of various ethnic groups show discrepancies. Studies have shown that the variety of *Lactobacillus* species in the vagina is limited to three to seven

species and strictly depends on the population of the studied women (Pavlova et al., 2002; Jin et al., 2007). Studies of Polish women indicated that their vaginal regions are most frequently colonized by three species of *Lactobacillus* (Pytka et al., 2019), with *L. crispatus*, *L. gasseri*, and *L. jensenii* being the most frequent. However, vaginal microbiota of Swedish healthy women is dominated in 78% by single LAB species. The most prevalent are: *L. crispatus, L. gasseri, L. iners* and *L. jensenii* (Vasquez et al., 2002). All isolated vaginal *Lactobacillus* strains identified in German women belonged to three species: *Lactobacillus crispatus* (56%), *Lactobacillus jensenii* (26%), and *Lactobacillus gasseri* (18%) (Hütt et al., 2016). Researchers from Turkey obtained similar results. They reported that, among 56 identified species 32% belonged to *L. crispatus*, 30% to *L. gasseri*, and 13.8% to *L. jensenii* (Eryilmaz et al., 2018). In an Italian study, 261 vaginal *Lactobacillus* species were detected, with *L. gasseri* (28%), *L. salivarius* (20%), *L. crispatus* (18%), *L. helveticus* (13%), *L. fermentum* (10%), and *L. rhamnosus* (10%) being the most common (Pino et al., 2019). The prevalence of *L. crispatus* and *L. iners* in vaginal was found to be lower in German females (42.4%), Indonesian population (45.0%), Kenyan women (34.4%) and Afro-Caribbean women (26.1%) compared to African American women (91.8%) (Roachford et al., 2022). Similarly, Ravel et al. (2011) observed that vaginal bacterial communities in North American women representing four ethnic groups (white, black, Hispanic, and Asian) were dominated by species of *Lactobacillus*. *Lactobacillus* groups I, II, III, and V were identified in 80.2 and 89.7% of Asian and white women, respectively, however, in only 59.6 and 61.9% of Hispanic and black women, respectively. Moreover, it was revealed that community group IV (diverse group) was overrepresented in Hispanic (34.3%) and black (38.9%) women in comparison to Asian (17.6%) and white (9.3%) women which suggest that such vaginal bacterial communities appear normal in black and Hispanic women (Ravel et al., 2011). The higher prevalence of communities not dominated by *Lactobacillus* sp. (cluster IV) in Hispanic and black females could be associated with differences in vaginal pH (5.0 ± 0.59 in Hispanic) and (pH 4.7 ± 1.04 in black females) in comparison to Asian (pH 4.4 ± 0.59) and white (pH 4.2 ± 0.3) women. Therefore, it appears that vaginal microbiome can change under factors such as glycogen content in epithelial cells, pH, hormone levels, damage caused by sexual intercourse, type of contraception, age, and antibiotic therapy (Valenta, 2005). As commonly known, the v environment is acidic at a pH of 4–4.5. 93 species of the genus *Lactobacillus* producing lactic acid are responsible for creating such conditions, which hinder colonization by pathogens. Nevertheless, after menopause, when estrogen levels decrease, the vaginal microbiome changes, species diversity increases, and *Lactobacillus* spp. co-exist with other species. As a result, vaginal pH and susceptibility to vaginal infections increase (Miller et al., 2016).

This study also shows that HPV infection is not recognized by many females as important risk factor for cervical cancer. Only less than 50% of women in this study have ever been tested for HPV, and none of them have been vaccinated against it. Persistent oncogenic HPV infections are essential for cervical oncogenesis. Studies indicate the involvement of the vaginal microbiota in the persistence or resolution of HPV infection. In a study on twins, Lee et al. (2013) showed that, when one of the twins is HPV-positive, there is a significant change in the vaginal microbiota, an increase in species diversity, and a decrease in the number of *Lactobacillus* spp. bacterial cells, compared with the healthy twin. Mitra et al. (2016) showed that the increasing diversity of the vaginal microbiome and the decreasing number of *Lactobacillus* cells are associated with the progression of cervical pathological changes.

The study was limited by the random selection of the study population and the limited possibility of extrapolating the results to the general population. We also did not include patients after HPV vaccination, which is not a very popular vaccination in women over 18 years of age in Poland. Further research is needed, particularly to determine the composition of the vaginal microbiota in women who have been vaccinated against HPV. Next limitation could be the use the assay based on qPCR amplification of a species-specific genetic region of the relevant microbe. Then, some species reported in other studies carried out with the use of next generation sequencing technique were not detected, i.e., *L. iners* or *L. jensenii*. Although the study has limitations, the results have the potential to be implemented in practice. There are increasing numbers of reports that vaginal application of certain dietary supplements can affect the vaginal microbiome in patients with abnormal cytology, resulting in regression of abnormal cytology and a return to normal cytology. This could be related to the influence of the vaginal microbiome bacteria on HPV or due to another mechanism. Determining the exact composition of the vaginal microbiome of healthy women in Poland might influence the use of this knowledge in the production of these preparations.

5. Conclusion

The data obtained in this study suggest that two *Lactobacillus* species (*L. gasseri* and *L. crispatus*) were predominant in the vaginal microbiota of the studied population of Polish women, regardless of smear test results. In this study, patient age and place of residence were associated with the diversity of *Lactobacillus* spp. in the vaginal microbiota. The abundance of *L. delbrueckii* in the vaginal microbiota increased, whereas that of *L. gasseri* decreased, with age. *L. acidophilus* and *L. fermentum* were significantly more frequently detected in women living in rural versus urban areas. The degree of dysplastic changes in the endothelium or the presence of a group of atypical cervical stratified epithelial cells was not associated with significant changes in the studied vaginal bacteria. A similar lack of significant differences in the qualitative and quantitative composition of the vaginal microbiota was also observed when analyzing associations with menopausal status, HPV infection, or vaginitis within 3 months before the study.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Available upon request. Requests to access these datasets should be directed to bbarczynski@poczta.onet.pl.

Ethics statement

The studies involving humans were approved by Bioethics Committee of Medical University in Lublin KE – 0254/174/2014. The

studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

KF: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. BB: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Project administration. RS: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – review & editing. AK: Writing – review & editing. AM: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. JK: Conceptualization, Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Validation, Visualization, Writing – review & editing. EW: Writing – review & editing. IK-G: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Investigation, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, 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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Metabolic flux reprogramming in *Mycobacterium tuberculosis*-infected human macrophages

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Metabolic fluxes are at the heart of metabolism and growth in any living system. During tuberculosis (TB) infection, the pathogenic *Mycobacterium tuberculosis* (Mtb) adapts its nutritional behaviour and metabolic fluxes to survive in human macrophages and cause infection. The infected host cells also undergo metabolic changes. However, our knowledge of the infected host metabolism and identification of the reprogrammed metabolic flux nodes remains limited. In this study, we applied systems-based ^{13}C-metabolic flux analysis (MFA) to measure intracellular carbon metabolic fluxes in Mtb-infected human THP-1 macrophages. We provide a flux map for infected macrophages that quantified significantly increased fluxes through glycolytic fluxes towards pyruvate synthesis and reduced pentose phosphate pathway fluxes when compared to uninfected macrophages. The tri carboxylic acid (TCA) cycle fluxes were relatively low, and amino acid fluxes were reprogrammed upon Mtb infection. The knowledge of host metabolic flux profiles derived from our work expands on how the host cell adapts its carbon metabolism in response to Mtb infection and highlights important nodes that may provide targets for developing new therapeutics to improve TB treatment.

KEYWORDS

tuberculosis, immunometabolism, *Mycobacterium tuberculosis*, fluxomics, human macrophages

Introduction

Tuberculosis (TB) remains a significant global health concern. COVID-19 pandemic negatively impacted TB diagnosis, treatment, and control (Pai et al., 2022). TB continues to spread, resulting in 10.6 million new cases in 2021 and 1.6 million deaths, reversing the years of slow decline in TB cases. The emergence of drug-resistant TB and the ineffectiveness of the current treatments pose a serious public health threat (World Health Organization, 2022). It is therefore urgent to identify new targets for drug development.

The causative agent of TB, *Mycobacterium tuberculosis* (Mtb) is a highly adaptable pathogen. Macrophages, the phagocytic innate immune cells, are one of the primary residing sites for Mtb (Howard and Khader, 2020). The interactions between host macrophages and Mtb are a primary determinant of the outcome of infection (Kumar et al., 2019; Howard and Khader, 2020). We know that the pathogenic Mtb acquires multiple nutrient sources including amino acids and

cholesterol from the host macrophages for intracellular replication (Pandey and Sassetti, 2008; Beste et al., 2013; Borah et al., 2019a,b). Thus, Mtb relies on host cell metabolites for its growth and survival during infection. Whilst Mtb's *in vitro* and *in vivo* metabolism has been extensively investigated, our understanding of the infected host cell metabolism remains underexplored.

Macrophage polarisation into the M1 pro-inflammatory or M2 anti-inflammatory phenotype is tightly linked to cellular metabolism (Howard and Khader, 2020). M1 macrophages use glycolysis for energy production, whiilst M2 macrophages rely on mitochondrial oxidative phosphorylation and fatty acid oxidation (Howard and Khader, 2020). Metabolic reprogramming in macrophages is important to regulate immune functions such as the production of cytokines and antimicrobial responses to eliminate Mtb infection (Kumar et al., 2019). Upon Mtb infection, macrophages acquire a M1-like phenotype with increased glycolysis and increased production of proinflammatory cytokines (Shi et al., 2016; Kumar et al., 2019; Howard and Khader, 2020). Previous studies have demonstrated that Mtb infection led to a metabolic switch to aerobic glycolysis in infected macrophages, like the Warburg effect described in cancer (Gleeson et al., 2016; Sheedy and Divangahi, 2021). The increase in glycolysis was accompanied by a downregulation of the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (OXPHOS) (Shi et al., 2016). Hypoxia-inducible factor 1 (HIF-1α) has gained significant interest due to its role in macrophage polarisation and regulation of glucose metabolism (Wang et al., 2017; Terán et al., 2022). Lactate production through the oxidation of glycolytically-derived pyruvate was significantly elevated in Mtb infected macrophages and mice (Shin et al., 2011; Shi et al., 2016). Mtb infection also induced host cell mitochondrial metabolic reprogramming. Mtb infection downregulated mitochondrial bioenergetics in THP-1 and human monocyte-derived macrophages (Cumming et al., 2018). An Mtb effector protein encoded by Rv1813c altered host mitochondrial function by inhibiting cytochrome c release from mitochondria and causing delayed apoptosis (Martin et al., 2023). Glutamine and arginine amino acid metabolism are important in regulating macrophage polarisation (Kim et al., 2020; Jiang et al., 2022). Immunometabolism is therefore an important research area that has received growing interest over the recent years to understand infected host metabolic reprogramming and develop innovative host-directed therapeutics (HDTs). To develop effective HDTs, it is crucial to know precisely which metabolic nodes are reprogrammed in infected macrophages. For example, statins are proposed adjuvants, but their molecular basis for anti-TB activity needs to be established (Dutta et al., 2020). Host cell glycolysis is an attractive target (Kim et al., 2020). However, glycolysis is central for cellular metabolism and for fuelling anti-TB responses, so inhibition or elevation of all or specific glycolytic nodes or enzymes will need to be carefully considered. Although some progress has been made to identify overall changes in the metabolic pathways of infected cells, there is still no information on the intracellular fluxes that drive pathway activity.

Metabolic fluxes are at the heart of driving cellular metabolism and metabolic reprogramming (Borah et al., 2021b). The intracellular carbon fluxes, core to sustaining cellular functions have not yet been measured. In this study, we applied fluxomics using ^{13}C-Metabolic Flux Analysis (MFA) to measure intracellular carbon metabolic fluxes of Mtb infected human macrophages. Our aim was to establish which fluxes or nodes in central carbon metabolism involving glycolysis, pentose phosphate pathway (PPP) and tricarboxylic acid (TCA) cycle were significantly reprogrammed upon Mtb infection. Here we provide the first measurements of carbon metabolic fluxes of Mtb-infected macrophages and highlight significantly reprogrammed fluxes that could be further investigated for potential HDT development.

Materials and methods

Model organisms

Mycobacterium tuberculosis (Mtb) H37Rv was the mycobacterial strain used in the study (Borah et al., 2019a,b). The THP-1 monocytic cell line was used as the human macrophage model used in this work (Borah et al., 2019a,b).

Mycobacterium tuberculosis H37Rv growth

Mtb H37Rv was cultured using Middlebrook 7H11 agar (Merck) and Middlebrook 7H9 broth (Merck) containing 10% (vol/vol) oleic acid-albumin-dextrose-catalase enrichment medium supplement (OADC) (Becton Dickenson) and 0.5% (vol/vol) glycerol (Merck). Mtb was streaked onto brain heart infusion agar (Merck) plates to check the purity of cultures prior to infection assays.

THP-1 cell culture

THP-1 monocytic cell line was grown in RPMI 1640 growth media (Merck) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Merck) at 37°C, 5% CO_2 and 95% humidity. For ^{13}C labelling experiments, modified RPMI growth media (Gibco™ 11,879,020) without glucose was prepared by adding 100% [U-$^{13}C_6$] glucose (w/v, CK Isotopes).

[U-$^{13}C_6$]glucose isotopic labelling assays

Isotopic labelling experiments were conducted by growing THP-1 macrophages in RPMI media containing 100% [U-$^{13}C_6$]glucose. Briefly, monocytes were grown in unlabelled RPMI media followed by differentiation into macrophages for three days using 50 nM Phorbol 12-myristate 13-acetate (PMA) in 175 cm^2 tissue culture flasks and in 6 well plates. Around 3×10^7 and 1×10^6 macrophages were cultivated in 175cm^2 flasks and 6 well plates, respectively.

Infection of THP-1 macrophages with Mtb H37Rv

THP-1 monocytes were differentiated into macrophages using PMA in RPMI media containing 100% [U-$^{13}C_6$]glucose as described in the previous section. On the day of infection, macrophages were cultivated either in 175 cm^2 tissue culture flasks or 6 well plates and washed with phosphate buffered saline (PBS) supplemented with 0.49 mM Mg2+ and 0.68 mM Ca2+ (PBS^+) three times prior to infection (Borah et al., 2019a). Mtb broth cultures were grown in 7H9

media with 10% OADC and 0.5% glycerol. Exponentially growing Mtb cultures were washed three times with PBS and added to macrophages at a multiplicity of infection (MOI) of 5. Macrophages infected with Mtb were incubated at 37°C, 5% CO_2 and 95% humidity for 3–4 h, followed by washing the macrophages with PBS^+ three times. Fresh media with 100% [U-$^{13}C_6$]glucose was added to infected macrophages and left for 48 h prior to cell harvest for metabolomics and isotopomer analysis. Uninfected macrophages were washed and incubated for 48 h in 100% [U-$^{13}C_6$]glucose containing RPMI as controls.

Metabolomics and ^{13}C isotopic labelling analysis

After 48 h of infection, uninfected and infected macrophages cultivated in 6-well plates were washed twice with PBS and cells were scraped. Cells were spun at 300 g for five minutes. 6 M hydrochloric acid (HCl) was added to the cells and boiled for 10 min (Borah et al., 2019a). Amino acid hydrolysates were prepared from cell lysates by incubating the lysates in 6 M HCl at 100°C for 24 h. Gas-chromatography mass spectrometry (GC–MS) metabolomics was performed to measure amino acid pool sizes and for mass isotopomer analysis. Amino acid hydrolysates were dried and derivatized using pyridine and tert-butyldimethyl silyl chloride (TBDMSCl) (Merck) (Borah et al., 2019a). Amino acids were analyzed using a VF-5 ms inert 5% phenyl-methyl column (Agilent Technologies) on GC–MS. Mass spectra were extracted using chemstation GC–MS software. Mass spectrometry datasets were corrected for natural isotope effects using the Isotopomer network compartmental analysis (INCA) platform (Young, 2014; Borah et al., 2021a). Average ^{13}C in an amino acid was calculated from the fractional abundance of the mass isotopomers in the amino acid fragments. The corrected mass isotopomer distributions (MIDs) for non-essential amino acids and organic acids were included into the macrophage metabolic model for flux estimation using ^{13}C-MFA.

Biomass measurements

Biomass measurements were calculated for uninfected and infected macrophages. 3×10^7 macrophages cultivated in 175 cm2 tissue culture flasks were used for biomass assays. For calculating dry cell weight, macrophage samples were distributed into eppendorfs, freeze dried and weighed. Dry cell pellets were used to measure protein using BCA (bicinchoninic acid) protein determination kit (Merck), lipids using lipid Assay Kit (Abcam ab242305), total DNA using Qubit dsDNA BR Assay (Fisher 10,146,592), total RNA using QUANT-IT RNA BR assay kit (Fisher 10,266,793) and carbohydrates using carbohydrate kit (Merck, MAK104). Amino acids were quantified using GC–MS method as described in the previous section.

^{13}C-MFA

Metabolic flux estimations were performed using INCA, version 2.2 (Young, 2014). The MIDs included in the model for flux estimation are provided in Supplementary File S2. Uninfected and infected macrophages were at pseudo-steady state (metabolic and isotopic) after 48 h of ^{13}C-labelling and Mtb-infection (Beste et al., 2013; Borah et al., 2019a). Fluxes were estimated using a non-linear weighted least squares fitting approach to calculate net and exchange flux distributions that were the best-fit for generation of the isotopic labelling data and biomass constraints (Young, 2014; Borah et al., 2021a). A random initial guess and multistart optimisation approach with 200 restarts was used for flux estimations. Flux profiles with the minimum statistically acceptable SSR were considered the best-fit. The goodness of fit of the flux maps was assessed by comparing the simulated and experimental measurements. The uncertainties in the flux estimations were checked using the Monte Carlo analysis function built into INCA. The confidence intervals and frequency distribution plots were calculated using Monte Carlo analysis, with significance level $\alpha = 0.05$, 100 trials per iteration and a relative error tolerance of 0.005.

Statistical analysis

Statistical analysis including chi-square test and Monte Carlo analysis was performed using INCA and t-tests were calculated using Graphpad version 8.2.1.

Metabolic modelling

Metabolic modelling of a macrophage was conducted using INCA, version 2.2 (Young, 2014). The isotopomer model consists of a central carbon metabolic network with 57 net flux reactions and 25 exchange fluxes (Supplementary File S1). Metabolic reactions include glycolysis, gluconeogenesis, the PPP, the TCA cycle, anaplerosis, biomass reaction and amino acid synthesis (glutamate, glutamine, proline, arginine, aspartate, asparagine, alanine, serine, glycine, cysteine, and tyrosine). The biomass reaction used to constrain the model was constructed from the experimentally measured biomass proportions of uninfected and infected macrophages (Figure 1). The network also consists of uptake reactions for glucose and amino acids: glutamine, alanine, glycine, serine, tyrosine, aspartate, asparagine, cysteine, arginine, glutamate, and proline. The glucose uptake flux was fixed at 100 and fluxes were estimated relative to the glucose uptake rate.

Results

^{13}C-MFA workflow for human macrophages

This section outlines the methodology for conducting ^{13}C-MFA in THP-1 macrophages (uninfected or infected with Mtb H37Rv) (Figure 1). [U-$^{13}C_6$]glucose was chosen as the isotopic substrate for the labelling experiment. THP-1 monocytes were differentiated into macrophages with PMA in RPMI growth media containing 100% [U-$^{13}C_6$]glucose for 72 h. We did not find any significant increase in ^{13}C incorporation into the biomass of macrophages when monocytes were cultivated in [U-$^{13}C_6$]glucose for 48 h prior to differentiation into macrophages. Hence, we chose to grow macrophages in ^{13}C labelling

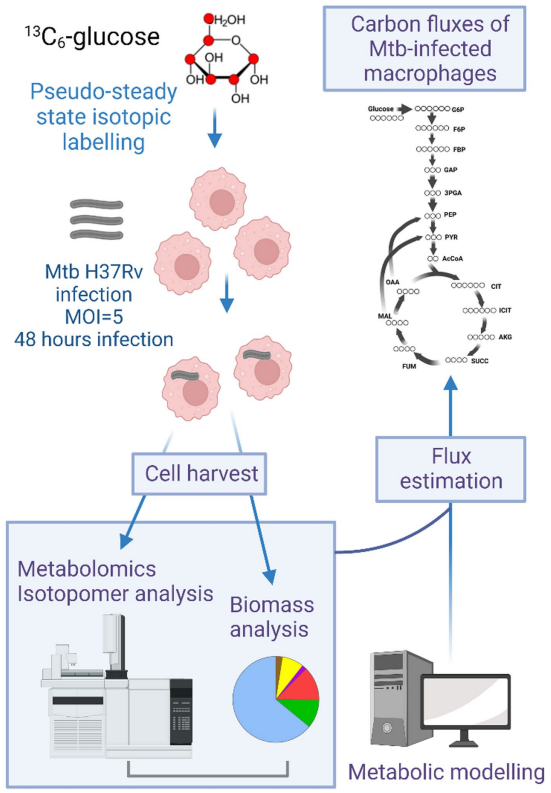

FIGURE 1
Overview of the method for conducting ^{13}C-metabolic flux analysis (MFA) in human THP-1 macrophages. Macrophages were grown in [U-$^{13}C_6$]glucose containing RPMI media for 72 h followed by infection with Mtb H37Rv for 48 h. After infection macrophages were harvested for metabolomics and isotopomer analysis using gas chromatography mass spectrometry (GC–MS). An isotopomer network model of human macrophages containing central carbon metabolic reactions was constructed. Metabolomics and isotopomer data were incorporated into the model and flux estimation was done using INCA.

media during differentiation and infection. Macrophages were uninfected (control) or infected with Mtb H37Rv for 48 h in ^{13}C labelling media. The infection period of 48 h was chosen as the pseudo steady-state labelling period in infected macrophages, as established from our previously published work (Beste et al., 2013; Borah et al., 2019a). Uninfected or infected macrophages were harvested after 48 h for metabolomics and isotopic labelling analysis. In parallel, biomass composition analyses were conducted for uninfected and Mtb-infected macrophages grown in unlabelled RPMI media. The biomass composition was used to construct the biomass equation that constrained the isotopomer metabolic network model which was constructed to include the central carbon metabolic reactions for glycolysis/ gluconeogenesis, pentose phosphate pathway (PPP) and tricarboxylic acid (TCA) cycle. The model was constructed based on published literature and databases including HumanCyc and KEGG (Amaral et al., 2011; Duckwall et al., 2013; Antoniewicz, 2018; Lagziel et al., 2019). The isotopomer data from the labelling experiments were incorporated into the model and computationally fitted using the MATLAB-based isotopomer network compartmental analysis (INCA) platform to compute the best-fit flux maps.

Mtb infection induces changes in biomass production by macrophages

Biomass measurements were used to constrain the metabolic model for flux estimations. We measured macromolecular biomass composition: nucleic acids, protein, lipids, and carbohydrates of uninfected and Mtb-infected macrophages (Figures 2A–E). Protein accounted for the highest proportion of cell dry weight (>50%), followed by lipids and carbohydrates (Figures 2F,G). There were no significant changes in total cellular lipids, protein, RNA and DNA upon Mtb infection. Total cellular carbohydrates in infected macrophages were significantly higher when compared to uninfected macrophages demonstrating the shift in carbohydrate synthesis upon Mtb infection (Figure 2E).

We quantified cellular amino acid pools using GC–MS metabolomics to identify changes induced in infected macrophages (Figure 2H). There were no significant differences in the pool sizes of essential amino acids (valine, leucine, isoleucine, methionine, threonine, lysine, histidine, tryptophan, phenylalanine) between the two groups as these are not synthesised in human cells but taken up from the growth media. Amongst the non-essential amino acids, proline and cysteine levels were significantly lower in infected macrophages. Cysteine is synthesised directly from serine; the pool size of cellular serine was over two-fold lower in infected macrophages. Aspartate and glutamate, the two primary precursors for amino acid biosynthesis did not change significantly upon Mtb-infection.

Mtb-infected macrophages show distinct ^{13}C isotopomer profiles of amino acids

In this section, we present the ^{13}C mass isotopomer distribution profiles for macrophage amino acids. The carbon backbone in amino acids is synthesised from metabolic intermediates generated from central carbon metabolism. The carbon backbone of alanine (Ala) and serine (Ser) are produced from glycolytic intermediates pyruvate and 3-phosphoglyceric acid, respectively. The ^{13}C distribution profile for Ser is strikingly different for infected macrophages; the proportional distribution of ^{13}C is highest in isotopomer family M3 for uninfected macrophages but M1 for infected macrophages (Figure 3). Quantitative analysis in Figure 4 shows that the fractional abundance or proportion of ^{13}C in M2 were significantly higher for infected macrophages. Glycine (Gly) and cysteine (Cys) which were synthesised from Ser also showed higher proportions of M2 and M3 in infected macrophages. This was expected as these three amino acids are synthesised from the same carbon precursor derived through glycolysis. The proportion of ^{13}C in Ala M3 is higher in uninfected macrophages (Figure 4). The differences in these amino acids indicate likely changes in carbon fluxes that generated respective metabolic precursors through glycolysis in infected macrophages. Tyrosine is derived from the PPP and glycolytic intermediates; the ^{13}C distribution profile is significantly different between uninfected and infected groups (Figures 3, 4) indicating changes in fluxes through these central pathways. The TCA cycle derived intermediates fumarate (FUM) and malate (MAL) and amino acids aspartate (Asp) and glutamate (Glu) exhibited quantitatively distinct proportional ^{13}C in infected macrophages (Figure 5). Although the total cellular pool sizes

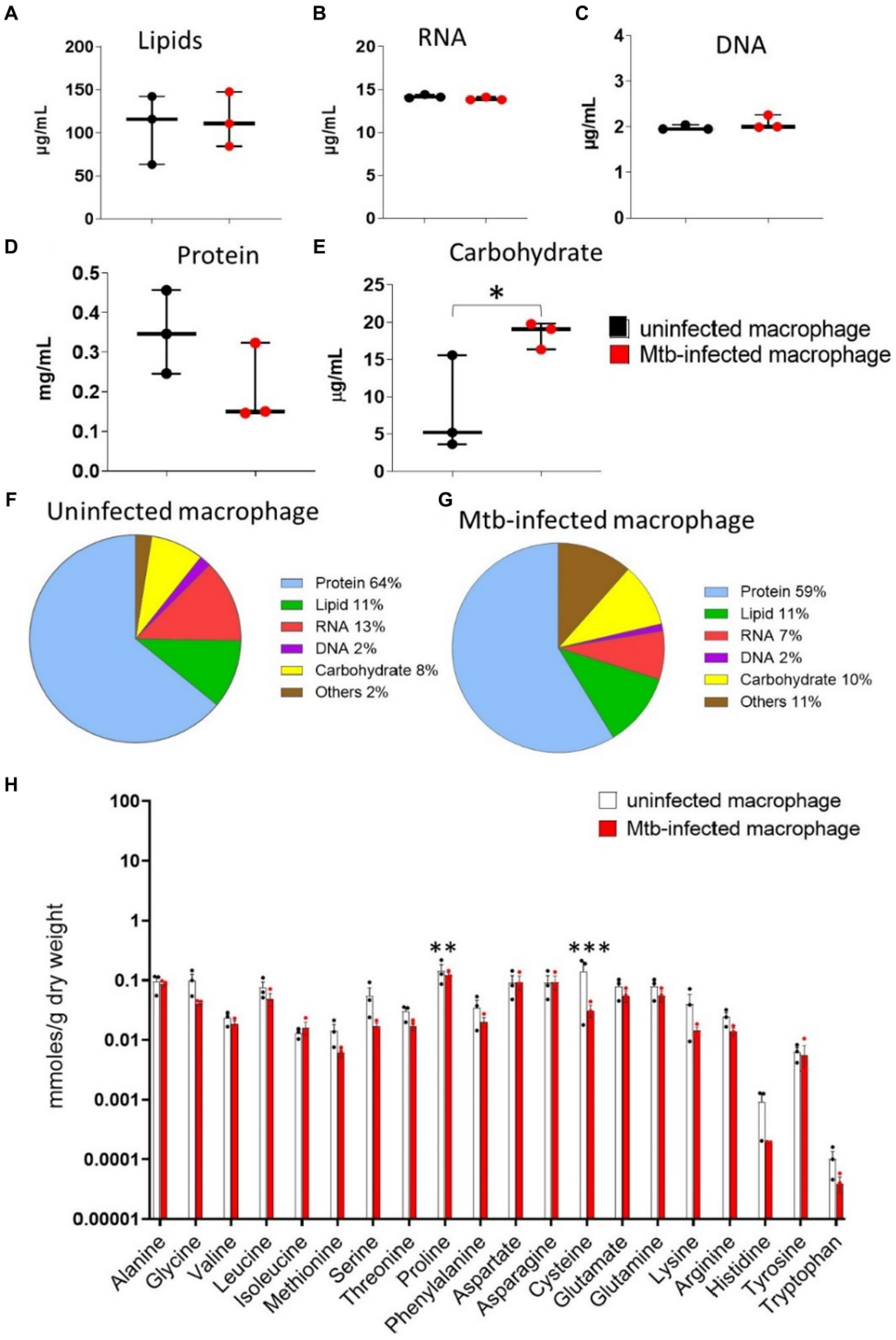

FIGURE 2
Macromolecular biomass composition measurements. Uninfected and Mtb-infected macrophages (post 48 h of infection) were harvested for measuring cell dry weight, **(A)** lipids, **(B)** RNA, **(C)** DNA, **(D)** protein and **(E)** carbohydrates. **(F,G)** Show the proportional biomass composition in uninfected and infected macrophages. Values are mean ± standard error of the mean (SEM) from three independent infections. Statistical significance is determined without correction for multiple comparisons, with alpha = 0.05; * indicates statistical significance, *, $p < 0.05$. **(H)** Cellular amino acid pool sizes for uninfected and Mtb-infected macrophages. Quantification was determined using GC–MS metabolomics. Values are mean ± standard error of the mean (SEM) from three to four independent infections. Statistical significance determined without correction for multiple comparisons, with alpha = 0.05; * indicates statistical significance; **, $p < 0.005$; ***, $p < 0.0005$.

of Asp., Glu and Tyr showed no significant differences between uninfected and infected macrophages, the ^{13}C isotopomer analysis was distinct between the two groups; this confirms that the measurement of pool sizes alone may not be an accurate indicator of changes in carbon fluxes through a pathway and ^{13}C isotopomer analyses could provide more detailed information. The mass isotopomer distribution

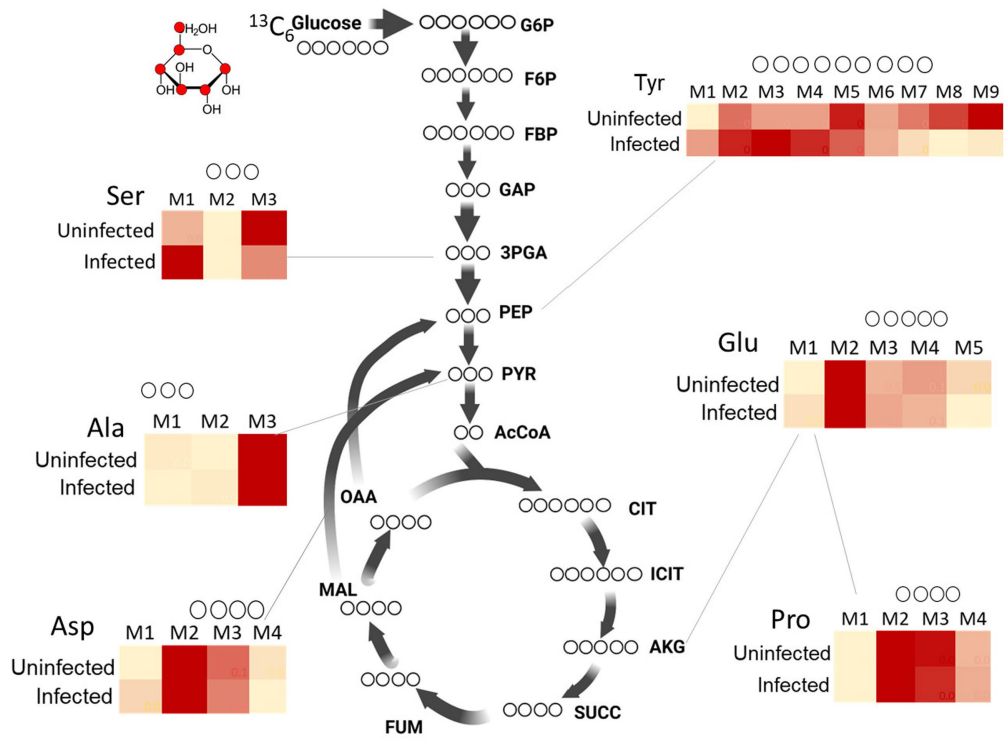

FIGURE 3
^{13}C isotopomer profiles of amino acids from uninfected and Mtb-infected macrophages. Comparison of ^{13}C distribution between uninfected vs. infected macrophages are shown for Ser (serine), Tyr (tyrosine), Ala (alanine), Asp (aspartate), Glu (glutamate) and Pro (proline). M1, M2, M3....M9 are carbon mass isotopomers. Values are mean ± standard error of the mean (SEM) from three independent infections.

profiles obtained from the labelling experiments were incorporated into the metabolic model to estimate carbon fluxes through the central metabolic network, which are presented in the next section.

Carbon metabolic flux phenotype of uninfected and Mtb-infected macrophages

Metabolic fluxes for central carbon metabolism (CCM) were quantified using ^{13}C-MFA (see Figure 1 for the overview of the steps for MFA). The details of reactions included in the isotopomer network model of a macrophage are provided in Supplementary File S1. Fluxes were resolved for the pathways involved in CCM: glycolysis/gluconeogenesis, PPP, and the TCA cycle (Supplementary File S3). The flux maps for uninfected and infected macrophages are shown in Figure 5. Fluxes were quantified relative to the glucose uptake rate set arbitrarily at 100. The uptake of glucose in macrophages has been reported to be mediated by GLUT1, a member of the SLC2 transporter family (Nonnenmacher and Hiller, 2018). In the cytosol, glucose is converted to glucose 6 phosphate (G6P) by hexokinase (Nonnenmacher and Hiller, 2018). Flux from G6P was partitioned to glycolysis via fructose 6 phosphate (F6P) and to PPP via 6-phosphogluconic acid (PG6). Reactions R4 to R10 of glycolysis carried the highest carbon fluxes relative to the PPP and the TCA cycle. The uninfected and infected macrophages profile exhibited oxidative TCA cycle fluxes. The TCA cycle fluxes were relatively lower than that in glycolysis and the PPP. Anaplerotic carbon metabolism was modelled between phosphoenolpyruvate (PEP) and oxaloacetate (OAA) and between pyruvate (PYR) and malate (MAL). The cytosolic and mitochondrial pools of OAA and MAL were lumped together to improve flux resolution in these reactions. Anaplerotic reactions carried the lowest carbon flux relative to the rest of the central pathways. We compared the best-fit carbon flux estimates between uninfected and infected macrophages and described the significantly altered pathways in the next section.

Glycolytic and PPP carbon fluxes were altered in Mtb-infected macrophages

The statistical significance of the differences between the fluxes of Mtb-infected and uninfected macrophages was assessed using Monte Carlo analysis. The frequency distribution plots for glycolytic fluxes are shown in Figure 6 and Supplementary Figure S1. Glycolytic fluxes R4, R5, R6, R7, R8, R9 and R10 were significantly higher in infected macrophages. Glycolysis is a faster route for ATP production and generation of biosynthetic precursors, which are required to mount host defence responses against Mtb (Shi et al., 2016). We have selectively illustrated the statistical comparisons for fluxes R4, R6 and R10 in Figure 6. Flux R4 from G6P to F6P, the first step of glycolysis was significantly higher in infected macrophages. The plots for R4 are significantly different between uninfected and infected macrophages. For uninfected macrophages, the maximum probable and best-fit net flux value of R4 is 66.9, but for infected macrophages, this value is 94.7. Similarly, the plots for R6 (from fructose 1,6-bisphosphate (FBP) to glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone

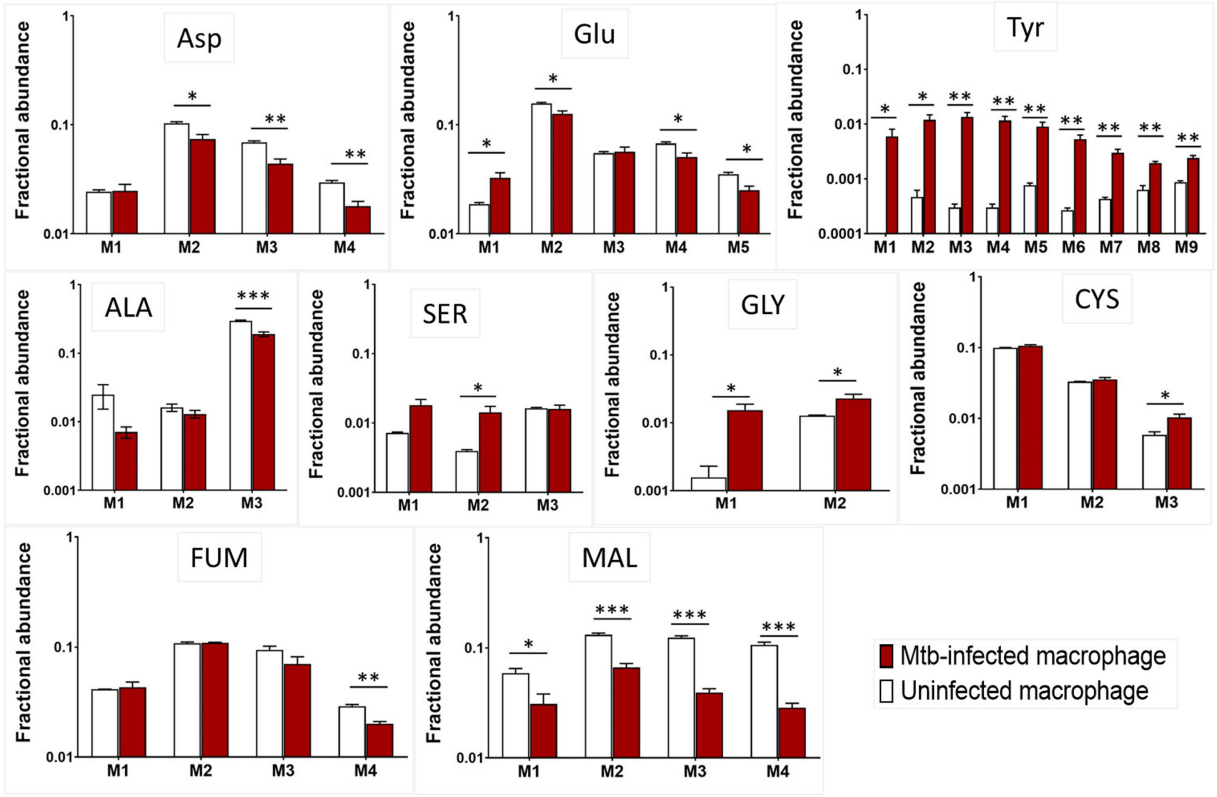

FIGURE 4
^{13}C incorporation in amino acids from uninfected and Mtb-infected macrophages. M1, M2, M3....M9 are carbon mass isotopomers shown for alanine (Ala), glutamate (Glu), tyrosine (Tyr), serine (Ser), glycine (Gly), cysteine (Cys), aspartate (Asp), fumarate (Fum) and malate (Mal). Values are mean ± standard error of the mean (SEM) from three independent infections.

phosphate (DHAP)) show the maximum probable best-fit flux of 98.2 for R6 in infected macrophages vs. 88.9 in uninfected macrophages. The comparisons for rest of the glycolytic fluxes R5, R7, R8 and R9 are shown in Supplementary Figure S1 which also shows significant changes in uninfected vs. infected macrophages. The plots for R10, the last step rate-limiting step of glycolysis from PEP to PYR catalysed by the pyruvate kinase enzyme show a maximum flux of 197.2 for infected macrophages vs. 188.4 in uninfected macrophages. Carbon flux from PYR is withdrawn for the synthesis of amino acids including ALA and lactate. The statistical resolution for these output fluxes was not refined, however the total flux withdrawn from PYR to synthesise ALA and lactate was higher in infected macrophages (195.19 in infected vs. 188.20 in uninfected macrophages).

The fluxes for both oxidative and non-oxidative branches of the PPP were significantly reduced in Mtb-infected macrophages. The plots for flux R11 (the first step of the PPP: G6P to PG6) and R12 (PG6 to ribulose 5-phosphate (RU5P)) show the best-fit flux value of 33 in uninfected macrophages vs. 5.2 in infected macrophages (Figure 7; Supplementary Figure S2). These oxidative PPP fluxes generate NADPH coenzymes that fuel cellular lipid synthesis. The fluxes through the non-oxidative branches of the PPP (R13, R14, R15, R16, R17, R18, R19) were also reduced in infected macrophages (Figure 7; Supplementary Figure S2). The plots for R13 and R14 in Figure 7 shows over a five-fold reduction of fluxes in infected macrophages and no overlap of the maximum value in the frequency distribution plots with that of uninfected macrophages.

We have demonstrated that infected macrophages have significantly higher glycolytic fluxes and reduced PPP fluxes. The flux partitioning between glycolysis and PPP was altered upon infection, channelling higher carbon catabolism through glycolysis. Glycolytic glucose catabolism generates adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) which are important coenzymes/cofactors for biological functions and energy production in cells (Figure 6). The increase in glycolytic fluxes resulting in higher production of ATP and NADH might be required to fuel the immune responses against Mtb. Targeting the reprogrammed fluxes in infection could help improve TB treatment. For instance, if we could boost glycolytic fluxes in infected cells through therapies this could enhance the elimination of Mtb.

Macrophages exhibited relatively low carbon fluxes through the TCA cycle

Fluxes through the TCA cycle were significantly lower compared to glycolysis. The TCA cycle generates co-enzymes that participate in the mitochondrial electron transport chain for ATP production. Macrophages are non-diving cells, so the relatively low TCA cycle fluxes indicate a low demand for carbon oxidation through this cycle and subsequent ATP production. Instead, the cellular energy and biosynthetic demand is met by glycolysis and the PPP fluxes. Thus, the TCA cycle fluxes are not the primary route for carbon oxidation in

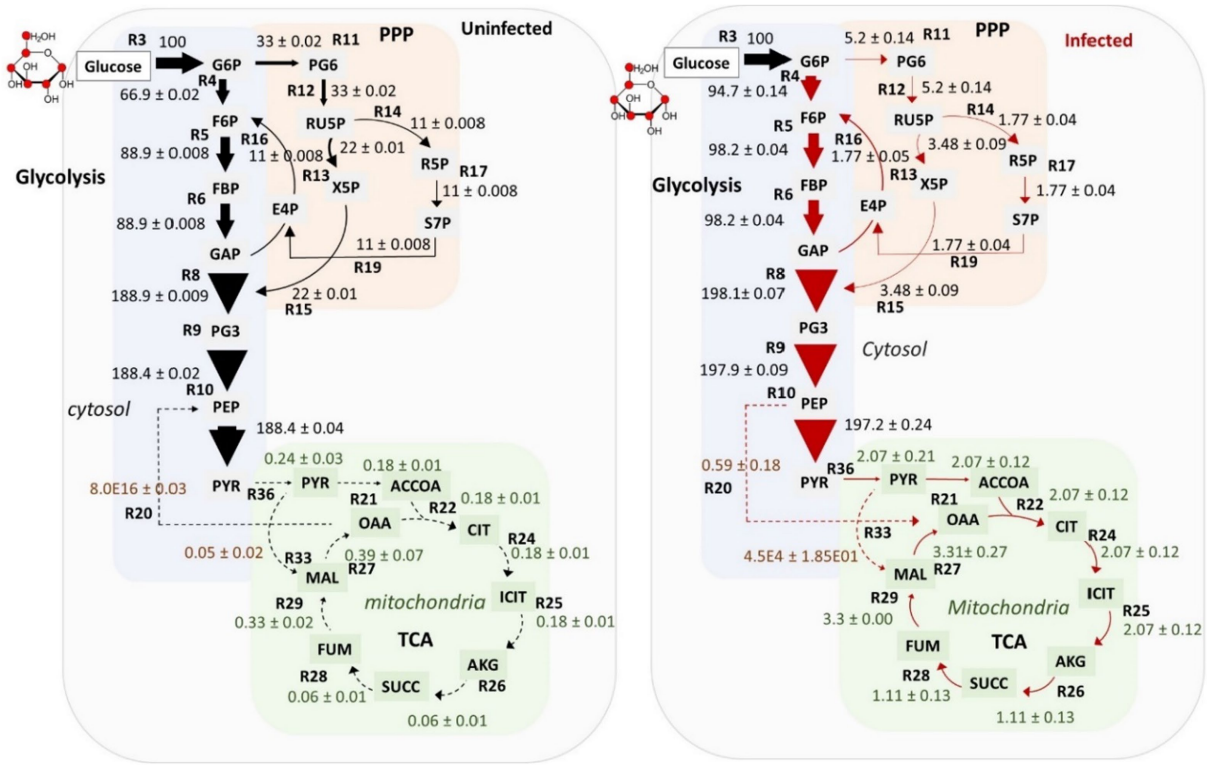

FIGURE 5
Flux maps of uninfected and infected macrophages. Fluxes were calculated relative to the glucose uptake rate set to 100. Fluxes are shown by arrows for the central carbon metabolic pathways: glucose/gluconeogenesis, pentose phosphate pathway (PPP), tri carboxylic acid (TCA) cycle and anaplerotic reactions. Fluxes are proportional to the thickness of the arrow. G6P (glucose 6-phosphate), F6P (fructose 6-phosphate), FBP (fructose 1,6-bisphosphate), GAP (glyceraldehyde 3-phosphate), PG3 (3-phophoglycerate), PEP (phosphoenolpyruvate), PYR (pyruvate), MAL (malate), OAA (oxaloacetate), SUCC (succinate), ACCOA (acetyl coenzyme A), ICIT (isocitrate), AKG (α-ketoglutarate), FUM (fumarate), PG6 (6-phosphogluconic acid), X5P (xylulose 5-phosphate), E4P (erythrose 4-phosphate), R5P (ribose 5-phosphate), RU5P (ribulose 5-phosphate) and S7P (sedoheptulose 7-phosphate). Fluxes are marked as R3, R4, R5....R36. See Supplementary File S3 for the list of all fluxes and their best-fit estimated values.

macrophages. Flux profiles show complete use of TCA cycle fluxes R21, R22, R24, R25, R26, R27, R28 and R29 in the oxidative phase (Figure 5). The frequency distribution plots in Supplementary Figure S3 shows no statistically significant differences in the TCA cycle and anaplerotic (malic enzyme and PEP carboxykinase) fluxes between uninfected and infected macrophages although the net fluxes were over 10 times higher in infected macrophages. These fluxes were statistically less well-resolved as compared to glycolysis and the PPP fluxes probably because of the choice of the ^{13}C-substrate. Here we chose glucose as the substrate, which is primarily utilised through glycolysis and the PPP, and therefore, these fluxes are statistically well-resolved.

Amino acid fluxes were reprogrammed in infected macrophages

We also quantified the carbon fluxes for non-essential amino acid biosynthesis (Figure 8). The TCA cycle and glycolysis are the primary sources of metabolic precursors required for the biosynthesis of amino acids. The TCA cycle-derived aspartate, arginine, glutamate, and glutamine and PYR-derived alanine biosynthetic fluxes were higher in infected macrophages. Aspartate, glutamate, and glutamine are acquired by the pathogenic Mtb as carbon and nitrogen nutrient sources during intracellular growth (Beste et al., 2013; Borah et al., 2019a). The precise role of these amino acids in macrophage metabolism and immunity still needs further investigation. The increased flux to arginine synthesis can be explained by the involvement of this amino acid in macrophage metabolism and immune responses against Mtb (McKell et al., 2021). Proline and asparagine fluxes did not change in infected macrophages and tyrosine fluxes (not shown in Figure 8) were higher in infected macrophages. The fluxes to glycolytic intermediate PG3-derived serine, cysteine, and glycine synthesis were reduced in infected macrophages (Figure 8). We have previously demonstrated that serine from host macrophages is not available to intracellular Mtb (Borah et al., 2019a). Mtb with deletion in *de novo* serine biosynthetic pathway failed to survive in human THP-1 macrophages (Borah et al., 2019a). The reduced serine biosynthetic flux in host macrophages is a metabolic phenotype associated with proinflammatory M1 macrophages that restricts bacterial growth.

Discussion

Host-pathogen metabolic interactions are important in TB. The TB pathogen adapts its metabolism and acquires nutrients during growth in human macrophages (Beste et al., 2013; Borah et al.,

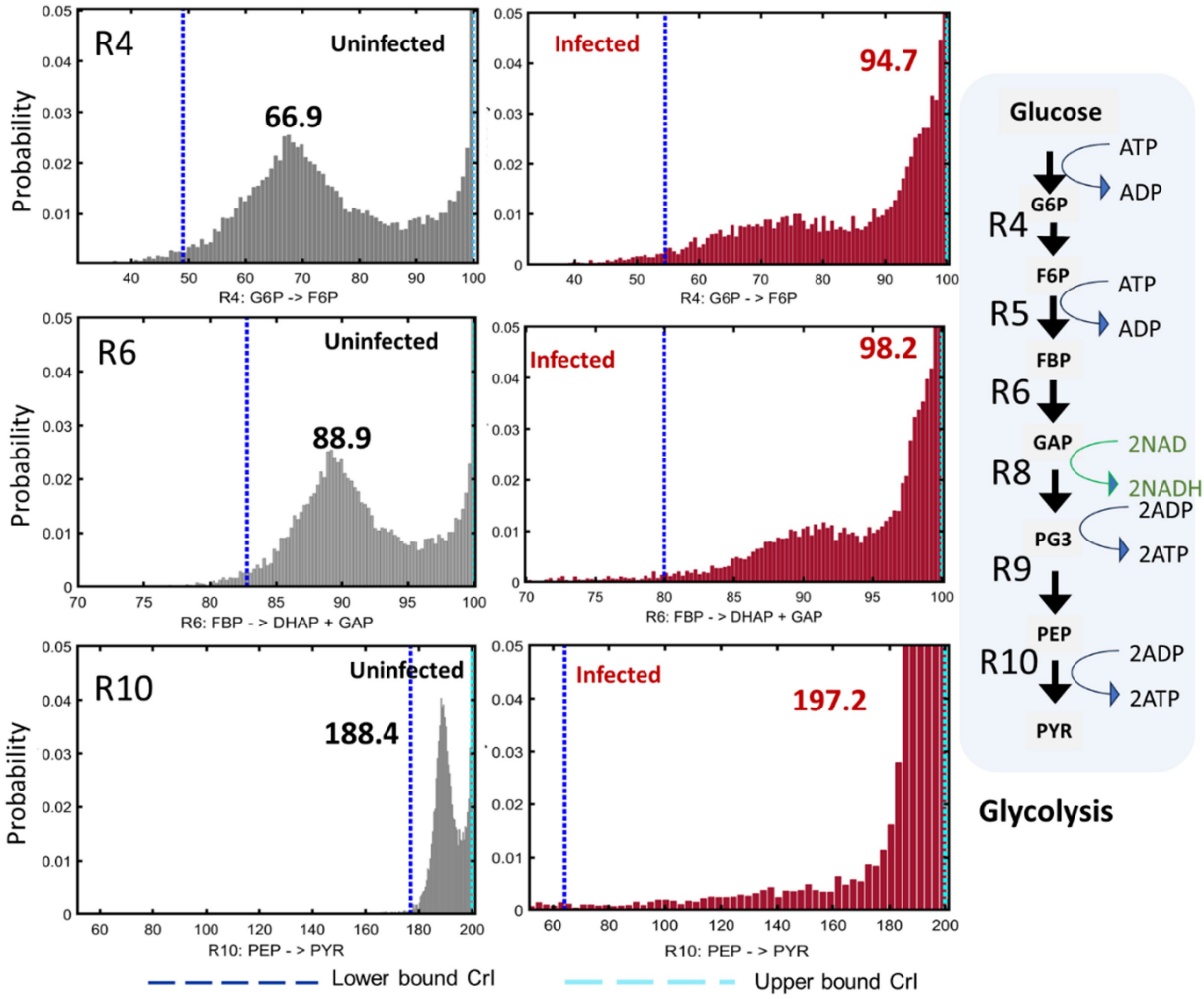

FIGURE 6
Glycolytic fluxes in uninfected and infected macrophages. The frequency distribution plots and upper and lower confidence intervals (CrI) for reactions R4 (G6P - > F6P), R6 (FBP - > DHAP + GAP) and R10 (PEP - > PYR) are shown. R4, R5....R10 are reactions in glycolysis. ATP, ADP, NAD and NADH are indicated next to the reactions that either consume or generate these coenzymes and cofactors. G6P (glucose 6-phosphate), F6P (fructose 6-phosphate), FBP (fructose 1,6-bisphosphate), DHAP (dihydroxyacetone phosphate), GAP (glyceraldehyde 3-phosphate), PEP (phosphoenolpyruvate) and PYR (pyruvate). ^{13}C isotopomer data of uninfected and infected macrophages (n = 3) were used for ^{13}C-MFA. Best-fit flux estimates were calculated relative to the glucose uptake rate (set at 100) using MATLAB-based isotopomer network compartmental analysis (INCA) platform. Monte Carlo simulations were performed using INCA to compute the frequency distribution plots and lower and upper confidence levels (CrI) for the best-fit fluxes.

2019a,b; Chandra et al., 2020). Previous work has reported that Mtb infection induces metabolic reprogramming in infected macrophages (Gleeson et al., 2016; Cumming et al., 2018). The metabolic reprogramming is intimately linked to the polarisation of macrophages to M1 and M2 functional phenotypes (Shi et al., 2016). Metabolic fluxes are core to facilitating this reprogramming, and the knowledge of the infected host's metabolic fluxes could ultimately provide new targets for therapeutic development. In this study, we applied ^{13}C-MFA to measure the intracellular carbon metabolic flux reprogramming of Mtb-infected human THP-1 macrophages.

We used human THP-1 macrophages as the *in vitro* infected host model for conducting ^{13}C-MFA. THP-1 monocytic cell line is a frequently used model in TB research and other areas. Other studies have investigated the relevance of THP-1 as a human macrophage model (Qin, 2012; Tedesco et al., 2018). The THP-1 macrophages showed similar mitochondrial respiratory and glycolytic extracellular acidification profiles as human monocyte-derived macrophages when infected with pathogenic Mtb H37Rv (Cumming et al., 2018). As our study is the first application of ^{13}C-MFA in human macrophages, we chose THP-1 as the macrophage model to minimise heterogeneity and variations between cell populations which exist with primary human monocyte-derived macrophages isolated from different individuals (Qin, 2012; Tedesco et al., 2018).

We quantified intracellular carbon metabolic fluxes of uninfected and Mtb-infected macrophages. Mtb infection leads to an increase in glycolytic fluxes, which is the characteristic metabolic phenotype of M1 proinflammatory microbicidal macrophages (Gleeson et al., 2016). Glycolytic fluxes are important to mount appropriate immune responses to control Mtb's growth in macrophages (Gleeson et al., 2016). Overexpression of glucose transporter-1 (GLUT1) has been demonstrated to increase glycolysis and proinflammatory cytokine interleukin (IL)-6) and tumour necrosis factor (TNF)-α production

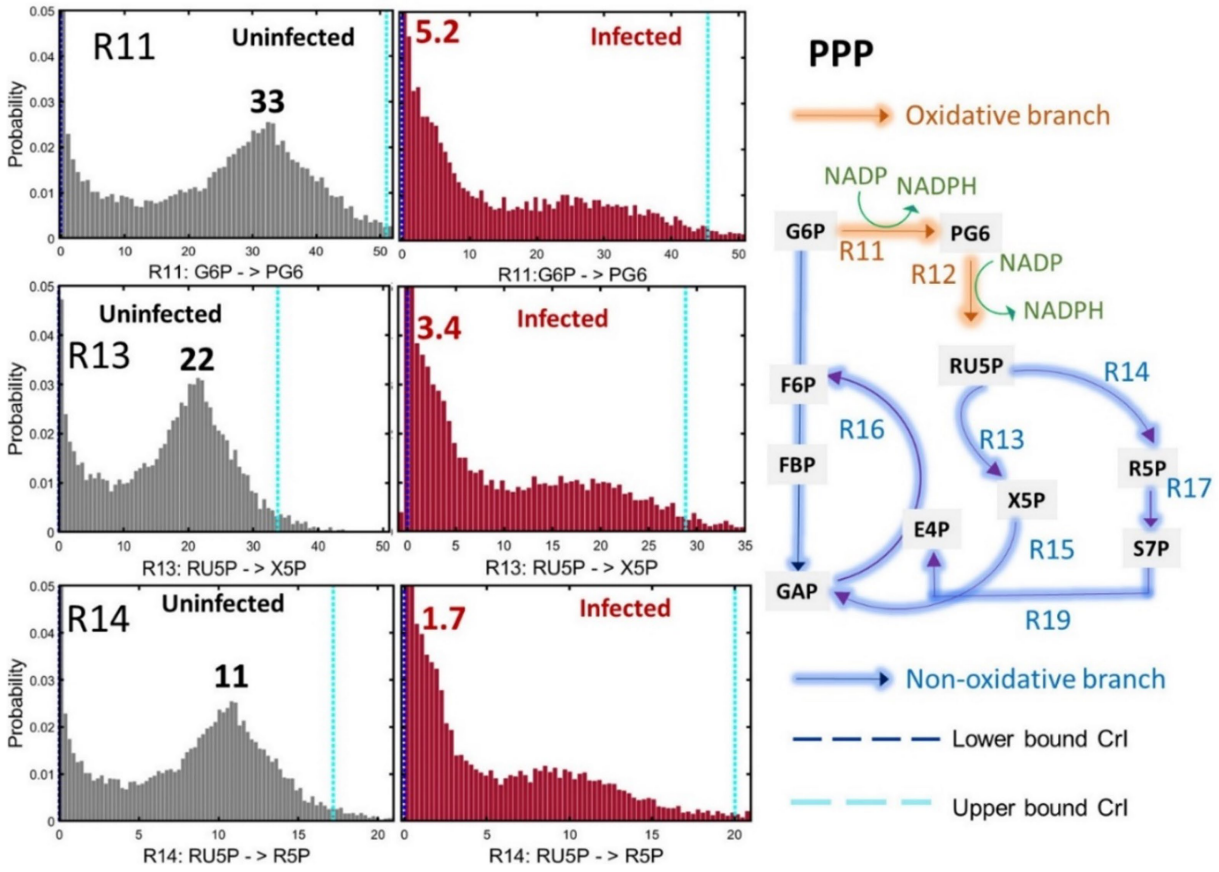

FIGURE 7
Pentose phosphate pathway (PPP) fluxes in uninfected and infected macrophages. The frequency distribution plots and upper and lower confidence intervals (CrI) for reactions R11 (G6P - > PG6), R13 (RU5P - > X5P) and R14 (RU5P - > R5P) are shown. Oxidative and non-oxidative reactions of the PPP (R11...R19) are shown in the pathway outline. NADP and NADPH indicated next to the reactions R11 and R12 are generated by oxidative branch of the PPP. G6P (glucose 6-phosphate), F6P (fructose 6-phosphate), FBP (fructose 1,6-bisphosphate), GAP (glyceraldehyde 3-phosphate), PG6 (6-phosphogluconic acid), X5P (xylulose 5-phosphate), E4P (erythrose-4-phosphate), R5P (ribose 5-phosphate), RU5P (ribulose 5-phosphate) and S7P (sedoheptulose-7-phosphate). 13C isotopomer data of uninfected and infected macrophages (n = 3) were used for 13C-MFA. Best-fit flux estimates were calculated relative to the glucose uptake rate (set at 100) using MATLAB-based isotopomer network compartmental analysis (INCA) platform. Monte Carlo simulations were performed using INCA to compute the frequency distribution plots and lower and upper confidence levels (CrI) for the best-fit fluxes.

(Gauthier and Chen, 2022). The shift of metabolic phenotype from oxidative phosphorylation (OXPHOS) to aerobic glycolysis in M1 macrophages is mediated by hypoxia inducible factor (HIF-1α) (Wang et al., 2017; Marín Franco et al., 2020; Terán et al., 2022). In extrapulmonary pleural TB, inhibition of HIF-1α and changes in macrophage phenotype from glycolysis to OXPHOS resulted in attenuation of microbicidal properties of infected macrophages and the consequent failure to restrict infection. Restoration of HIF-1α activity increased glycolysis in macrophages and in C57BL/6 mice resulting in better control of Mtb (Marín Franco et al., 2020). In addition to macrophages other immune cells, such as tissue-resident innate lymphoid cells and CD8+ T cells showed an increased metabolic dependency on glycolysis upon Mtb infection, which highlights the intimate link between glycolysis and immune responses across multiple immune cells (Russell et al., 2019; Corral et al., 2022).

The increased flux through glycolysis is directly linked to the increase in the total cellular carbohydrates of infected macrophages. The glycolytic flux profile of THP-1 macrophage is relevant to the metabolic changes measured in other *in vitro* (primary macrophages) and *in vivo* models of TB using other omic tools. Metabolite profiling using ^{1}H-NMR (nuclear magnetic resonance) spectroscopy in C57Bl/6 mice infected with pathogenic Mtb showed elevated levels of glucose and lactate indicating higher glycolytic fluxes upon Mtb infection (Shin et al., 2011). A different study demonstrated glycolytic differences in primary macrophages infected with virulent and non-virulent Mtb (Cumming et al., 2018). This study showed a reduced glycolytic extracellular acidification rate in Mtb-infected macrophages which contrasts with ours and other studies that demonstrate significant glycolytic dependency of the host when tested in *in vitro* macrophages and *in vivo* mice models. The differences in the observations could be attributed to the different experimental set up used. This study used an infection period of 18 h followed by 8 h of ^{13}C-glucose labelling, which is probably not enough to reach steady state labelling in these cells. Our ^{13}C-glucose labelling of Mtb-infected macrophages was conducted for 48 h which was established as the pseudo steady-state period where the cells have reached isotopic steady state labelling; there were no significant changes in ^{13}C-labelling

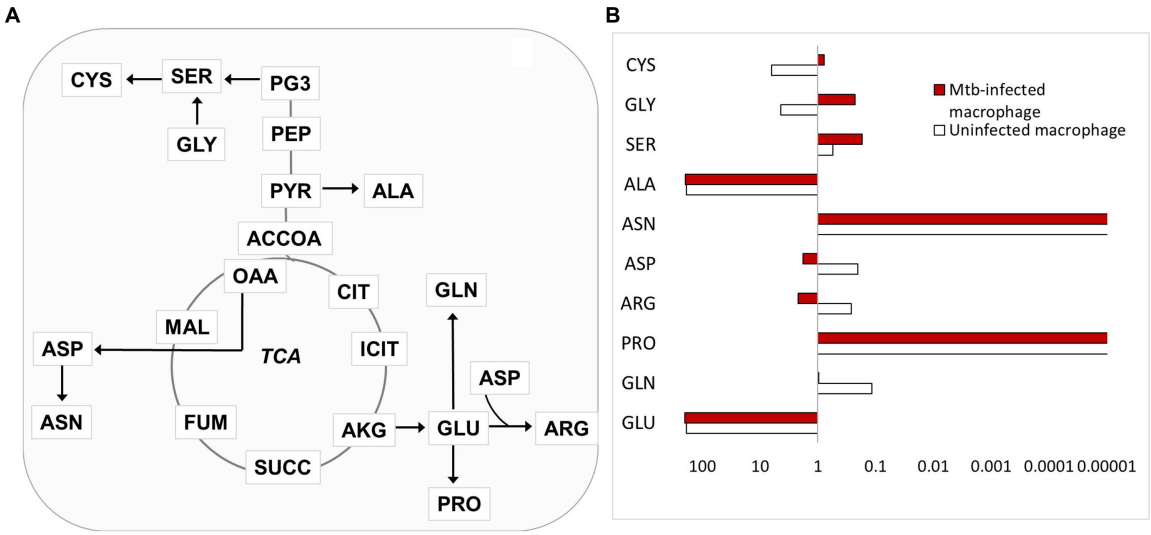

FIGURE 8
Carbon flux distributions in amino acid synthesis. **(A)** Outline of amino acid synthesis in macrophages. Serine (SER) is synthesised from PG3; cysteine (CYS) is synthesised from SER; glycine (GLY) is synthesised from SER, the arrow from GLY to SER indicates SER synthesis from GLY; alanine (ALA) is synthesised from PYR; glutamate (GLU) is synthesised from AKG; glutamine (GLN), proline (PRO) is synthesised from GLU; arginine (ARG) is synthesised from ASP and GLU. **(B)** Net fluxes relative to glucose uptake are shown for the amino acid metabolic fluxes represented in **A**.

after 48 h which indicate that the macrophages have achieved metabolic steady state (Borah et al., 2019a).

The glycolytic fluxes lead to pyruvate synthesis which is primarily utilised for lactate and/or alanine synthesis. We found that the total flux from pyruvate to lactate/alanine production was significantly higher in Mtb-infected macrophages. Others have also shown higher levels of lactate production in human alveolar and monocyte-derived macrophages, and murine bone marrow derived macrophages when infected with Mtb (Gleeson et al., 2016). Mtb uses host cell lactate during infection as a carbon substrate; this was demonstrated by the attenuation of an Mtb strain unable to oxidise lactate in primary macrophages (Billig et al., 2017). Thus, lactate is one of the key metabolites for host-pathogen interaction in TB but is a debatable target for HDT. Lactate can be used to refuel glycolysis and its synthesis is important to control Mtb's growth, so inhibiting lactate production in the host is not a potential target (Kiran and Basaraba, 2021). However, compounds that could target Mtb's lactate synthesis specifically could be a potential alternative to develop anti-TB therapies.

The higher fluxes through glycolysis were accompanied by reduced PPP fluxes in infected macrophages. The PPP produces NADPH and ribose 5-phosphate biosynthetic precursors required for cell proliferation, nucleotide metabolism and lipid synthesis. The reduced carbon fluxes through the PPP suggest the redistribution of a large proportion of carbon flux through glycolysis to generate energy for fuelling immune responses against Mtb whilst reducing the carbon flux to biomass production. The mitochondrial TCA cycle is a critical pathway to generate substrates and precursors for ATP production in a cell. M1 macrophages are characterised by a low dependency on the TCA cycle and OXPHOS. The mitochondrial energy metabolism was significantly reduced in macrophages and T-cells infected with Mtb (Cumming et al., 2018; Russell et al., 2019). Our flux maps show relatively low carbon fluxes through the TCA cycle in uninfected and infected macrophages. Although statistically insignificant, the net flux values for the TCA cycle in infected macrophages were higher, suggesting subsequent activity of the mitochondrial OXPHOS. A recent study using human macrophage and zebra fish model of TB established that Mtb infection increased mitochondrial OXPHOS which was required to prevent mitochondrial damage at early stages of infection (Pagán et al., 2022). Here we show that the TCA cycle fluxes are active in infected macrophages although it is not the primary carbon utilising pathway.

Macrophage amino acid fluxes were reprogrammed upon Mtb infection. Serine, glycine, and cysteine fluxes were reduced in infected cells; these three amino acids share a common biosynthetic pathway. Our previous work established that serine is not available to intracellular Mtb; a serine auxotroph of Mtb failed to survive in macrophages (Borah et al., 2019a). The fact that fluxes to serine synthesis was reduced in infected macrophages could be a strategy to limit nutrition to Mtb and restrict its growth. Serine is an important amino acid in cell metabolism; this amino acid is involved in one-carbon, glutathione, nucleotide, and coenzyme (NADH and ATP) metabolism (Gauthier and Chen, 2022). The role of this amino acid in TB needs to be further investigated as it can lead to the development of potential HDTs.

Mtb acquires aspartate, glutamine, and glutamate from host macrophages; these amino acids are both carbon and nitrogen sources for intracellular Mtb (Beste et al., 2013; Borah et al., 2019a). The fluxes for aspartate, glutamine and glutamate synthesis are elevated in infected macrophages. Glutamine was demonstrated to be important for M1 macrophage polarisation; inhibition of glutamine catabolism reduced macrophage polarisation when tested in Mtb-infected murine bone marrow-derived macrophages (Jiang et al., 2022). Aspartate and glutamate are central precursors for the synthesis of other amino acids, and they participate in central metabolism. However, the roles of these two amino acids in macrophage immunometabolism are unclear and need further assessment. Arginine derived from aspartate

and glutamate had elevated biosynthetic flux in infected macrophages. Arginine catabolism leads to the generation of nitric oxide which controls Mtb's growth and reduces tissue damage in TB granulomas (Duque-Correa et al., 2014). The flux for alanine synthesis was also higher in infected macrophages. Alanine of host macrophages is used by Mtb and incorporated directly into its biomass (Borah et al., 2019a). The levels of these amino acids were found to be higher in the lungs of Mtb-infected mice compared to the uninfected (Shin et al., 2011). The increased levels of free amino acids have been linked to the impairment of protein synthesis, i.e., amino acids are used for metabolic processes rather than protein anabolism (Shin et al., 2011). We found that the total cellular protein content was lower in infected macrophages; this is probably associated with the higher amino acid metabolic fluxes that are involved in driving immunometabolism and immune functions, but the precise link still needs to be understood.

Conclusion

In this study we present metabolic flux reprogramming of Mtb-infected human THP-1 macrophages. We conducted ^{13}C-MFA to derive carbon flux profiles that drive cellular metabolism and function. Glycolytic fluxes were significantly higher leading to increased pyruvate metabolism, and an overall increase in total cellular carbohydrate. The PPP fluxes were reduced upon infection. The TCA cycle-derived amino acid fluxes were higher and serine synthesis was reduced in infected macrophages. The Mtb-infected macrophage flux profile highlights the key nodes that are reprogrammed during infection and are the targets for further investigation. This may lead to the development of new therapies such as adjuvants to be used with antimicrobials to improve infection outcomes in TB patients.

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. 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

KB: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. LM: Formal analysis, Writing – review & editing. YX: Formal analysis, Writing – review & editing. DK: 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.2023.1289987/full#supplementary-material

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The splicing factor SR2 is an important virulence factor of *Toxoplasma gondii*

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Serine/arginine-rich (SR) proteins are key factors with important roles in constitutive and alternative splicing (AS) of pre-mRNAs. However, the role of SR splicing factors in the pathogenicity of *T. gondii* remains largely unexplored. Here, we investigated the role of splicing factor SR2, a homolog of *Plasmodium falciparum* SR1, in the pathogenicity of *T. gondii*. We functionally characterized the predicted SR2 in *T. gondii* by gene knockout and studied its subcellular localization by endogenous protein HA tagging using CRISPR-Cas9 gene editing. The results showed that SR2 was localized in the nucleus and expressed in the tachyzoite and bradyzoite stages. *In vitro* studies including plaque formation, invasion, intracellular replication, egress and bradyzoite differentiation assays showed that deletion of SR2 in type I RH strain and type II Pru strains had no significant effect on the parasite growth and bradyzoite differentiation ($p > 0.05$). Interestingly, the disruption of SR2 in RH type I ($p < 0.0001$) and Pru type II ($p < 0.05$) strains resulted in varying degrees of attenuated virulence. In addition, disruption of SR2 in type II Pru strain significantly reduced brain cyst burden by ~80% ($p < 0.0001$). Collectively, these results suggest that splicing factor SR2 is important for the pathogenicity of *T. gondii*, providing a new target for the control and treatment of toxoplasmosis.

KEYWORDS

***Toxoplasma gondii*, SR proteins, splicing factor SR2, alternative splicing, pathogenicity, CRISPR-Cas9**

1 Introduction

Toxoplasma gondii is an obligate intracellular apicomplexan parasite which infects many warm-blooded animals and humans. It is estimated to chronically infect one third of the world's population, causing public health and socio-economic impacts (Montoya and Liesenfeld, 2004; Milne et al., 2020). Although infection is asymptomatic in immunocompetent individuals, the reactivation of a dormant *T. gondii* infection in immunocompromised individuals such as HIV and organ transplant recipients may lead to severe and life-threatening consequences (Elsheikha et al., 2021). In addition, congenital infection can lead to miscarriages or mental and visual impairment (Smith et al., 2021). There is a clinical need to explore new targets for therapeutic development.

T. gondii has a remarkable ability to respond to and thrive in various environments. The success of *T. gondii* is partly facilitated by its ability to reprogram its gene expression at the transcriptional and post-transcriptional levels. Alternative splicing (AS) is a crucial cellular process which allows a single gene to produce multiple mRNA transcripts and, consequently, expand the protein repertoire encoded by the genome. AS plays vital roles in normal cellular function and development, and is widespread in human genome (Wang et al., 2008). Disruption or dysregulation of AS can lead to various diseases, including cancer, neurodegenerative diseases, cardiovascular diseases, and age-related diseases (Dlamini et al., 2015; Latorre and Harries, 2017; Du et al., 2021; Lv et al., 2021; Nikom and Zheng, 2023).

The serine/arginine-rich (SR) protein family, which contains one or two RNA recognition motifs (RRM) at the N-terminus and arginine/serine (RS)-rich domain at the C-terminus, is involved in the regulation of mRNA stabilization, translation, and splicing (Long and Caceres, 2009; Shepard and Hertel, 2009). The SR proteins and other splicing factors have been studied in several organisms using genetic editing approaches (Wang et al., 2016; Yuan et al., 2018). For example, disruption of the SR protein CeSF2/ASF in *Caenorhabditis elegans* results in growth defect and embryonic lethality (Longman et al., 2000). Mutation of SR-like RNA-binding protein slr1 in *Candida albicans* reduces its growth and filamentation, impairs its ability to damage host cells, and reduces the fungal virulence (Ariyachet et al., 2013). Ablation of SR-related protein RRM1 in *Trypanosoma brucei* results in cell cycle arrest and cell death (Levy et al., 2015).

Four SR splicing proteins (SR1, SR2, SR3, and SR4) are identified in *T. gondii* (Yeoh et al., 2015). Overexpression of SR3 causes growth defects and affects AS of over 1,000 genes in *T. gondii* (Yeoh et al., 2015). Given the important roles of SR proteins in *T. gondii* and other organisms, it is important to expand our understanding of the biological functions of other SR proteins in *T. gondii*. In this study, we investigated the biological roles of SR2 in *T. gondii* pathogenicity because its homologous protein in *Plasmodium falciparum* is essential for the parasite proliferation, and its overexpression affects the AS of *P. falciparum* genes (Eshar et al., 2012). CRISPR-Cas9 was used to establish knockout *T. gondii* strains, RH (type I) and Pru (type II), deficient in SR2. The resultant strains were used to identify the major phenotypic differences between the wild-type, the Δ*sr2*, and the complemented Δ*sr2*C strain to gain insights into the roles of SR2 in the parasite growth and pathogenicity. Our data showed that SR2 had no marked effect on the *in vitro* parasite growth and tachyzoite to bradyzoite differentiation, however it was significantly implicated in the virulence of *T. gondii* in mice.

2 Materials and methods

2.1 Mice

Specific-pathogen-free (SPF) female Kunming mice, between 6–8 weeks of age, were purchased from the Experimental Animal Center of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Lanzhou, China). All mice were acclimatized for one week prior to the study and had free access to water and food. All efforts were taken to minimize any suffering of the mice. Mice were immediately euthanized once they reach the humane endpoint. All experimental protocols were reviewed and approved by the Animal Research Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Permit No. 2022–009).

2.2 Host cells and parasite strains

The tachyzoites used in this experiment, including those of the wild-type strains (RHΔ*ku80* and PruΔ*ku80*, abbreviated as RH and Pru), knockout strains (RHΔ*ku80*Δ*sr2* and PruΔ*ku80*Δ*sr2*, abbreviated as RHΔ*sr2* and PruΔ*sr2*), complemented strains (RHΔ*ku80*Δ*sr2*C and PruΔ*ku80*Δ*sr2*C, abbreviated as RHΔ*sr2*C and PruΔ*sr2*C) and tagged strain (RH::SR2-6HA), were cultured by passage in human foreskin fibroblasts (HFFs, ATCC, Manassas, VA, United States). The cell cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% fetal bovine serum (FBS), 10 mM HEPES (pH 7.2), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C, humid atmosphere with 5% CO_2 as previously described (Wang et al., 2020; Liang et al., 2021). Tachyzoites were isolated from heavily infected HFFs using a 27-gauge needle and filtered through a 5-μm polycarbonate membranes.

2.3 CRISPR-Cas9 mediated C-terminus epitope tagging

C-terminus epitope tagging was performed to examine the subcellular localization of SR2 as previously described (Cao et al., 2019; Liang et al., 2021). Briefly, specific CRISPR-Cas9 plasmid (30 μg) targeting SR2 C-terminus (after the stop codon), and a 42 bp homologous arm (20 μg) flanking the 6 × hemagglutinin (HA) tag and DHFR fragment amplified from pLIC-6HA-DHFR plasmid were co-transfected into purified tachyzoites. After treatment with 3 μM pyrimethamine, positive clones were identified by Western blotting (WB) and immunofluorescence assay (IFA). All plasmids, primers and guide RNA used in the study are summarized in Supplementary Table S1.

2.4 Construction of SR2 knockouts

The CRISPR-Cas9 mediated homologous recombination was used to produce SR2 knockouts as previously described (Wang et al., 2020). Briefly, the target guide RNA in the template plasmid pSAG1::CAS9-U6::sgUPRT was replaced by a specific guide RNA of SR2 to complete the construction of SR2-specific CRISPR plasmid. The 5′ and 3′ HR regions of SR2 amplified from *T. gondii* genomic DNA and DHFR fragment amplified from pUPRT-DHFR-D plasmid were ligated to the pUC19 fragment amplified from the pUC19 plasmid using the Clone Express II one-step cloning kit (Vazyme, Nanjing, China). The SR2-specific CRISPR plasmid (30 μg) and 5'HR-DHFR-3'HR homologous fragment (20 μg) amplified from total ligated product were transfected into the freshly purified tachyzoites. After selection with 3 μM pyrimethamine, single clones were isolated by limiting dilution and identified by PCR.

2.5 Generation of SR2-complemented strains

To complement SR2, coding sequence was integrated into the uracil phosphoribosyl transferase (UPRT) locus by homologous recombination as previously described (Wang et al., 2020). SR2 promoter amplified from *T. gondii* DNA and coding sequence of SR2 amplified from the cDNA of RH strain were fused with a fragment containing 3 × HA tag, 3' UTR of DHFR, and a chloramphenicol acetyltransferase (Cat) cassette to produce complemented plasmids (pSR2::SR2::Cat). The corresponding fragments were amplified from pSR2::SR2::Cat plasmids, and co-transfected with the pSAG1::Cas9-U6::sgUPRT plasmid into the SR2 knockouts. Positive clones were selected by 20 μg/mL chloramphenicol acetyltransferase and identified using PCR and WB.

2.6 Immunofluorescence and Western blotting analysis

IFA was performed as previously described (Li D. et al., 2023; Li T. T. et al., 2023). Briefly, *T. gondii* infected samples were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton-100 for 20 min at room temperature. After blocking with 3% bovine serum albumin (BSA) for 2 h at 37°C, the samples were incubated with primary antibodies, including rabbit anti-IMC1 (1:500), mouse anti-HA (1:500) (Thermo Fisher Scientific, Waltham, MA, United States) for 2 h at 37°C, followed by secondary antibodies, including Alexa Fluor 488 goat anti-rabbit IgG (H + L) (1:500), Alexa Fluor 594 goat anti-mouse IgG (H + L) (1,500) (Thermo Fisher Scientific, Waltham, MA, United States), and FITC-*Dolichos Biflorus* lectin (DBL, Vector Laboratories) at 37°C for 1 h. The samples were visualized using Leica Confocal microscope system (TCS SP8, Leica, Germany).

For the WB analysis, purified tachyzoites were lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, United States) on ice for 1 h to extract total protein. The lysates were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane (Immobilon Millipore). Rabbit anti-aldolase (1:500), rabbit anti-HA (1:500), and goat anti-rabbit HRP (1,500) (Abcam, Cambridge, MA, United States) antibodies, 1% BSA working dilution, were used for WB analysis. ECL chemiluminescent reagent (Thermo Fisher Scientific, Waltham, MA, United States) was used to detect the signal and images were captured with Minichemi 610 chemiluminescent imager (Bio-Rad Laboratories, Hercules, CA, United States).

2.7 Plaque assay

To assess whether the absence of SR2 affects the lytic cycle of *T. gondii*, confluent HFFs grown on 12-well tissue culture plates (Thermo Fisher Scientific, Waltham, MA, United States) were infected by 500 freshly lysed tachyzoites per well for seven or nine days. After washing, five times with phosphate-buffered saline (PBS), HFFs were fixed with 4% PFA for 30 min and stained with 0.2% crystal violet for 20 min at room temperature. The size and number of plaques were examined with a scanner (Perfection V700 Photo) and analyzed by Image J.

2.8 Invasion assay

About 10^6 freshly egressed tachyzoites were allowed to invade HFFs for 30 min and fixed with 4% PFA for 20 min. The infected cells were subsequently incubated with antibodies, including primary antibody, mouse anti-SAG1 antibody (1:500), and secondary antibody, Alexa Fluor 594 goat anti-mouse IgG (H + L) antibody (1:500). After three washes with PBS, samples were permeabilized with 0.1% Triton-100 and sequentially stained with rabbit anti-GAP45 antibody (1:500) and Alexa Fluor 488 goat anti-rabbit IgG (H + L) antibody (1,500). IFA was used to examine the parasite's invasion efficiency by evaluating the ratio of intracellular tachyzoites (green-stained minus red-stained) and total tachyzoites (green-stained).

2.9 Intracellular replication assay

To evaluate the role of SR2 in the replication of *T. gondii*, 10^5 freshly egressed tachyzoites were allowed to infect HFF monolayers on 12-well tissue culture plates for 1 h, and then washed with DMEM to remove extracellular tachyzoites. The plates were incubated at the same conditions for another 23 h, fixed with 4% PFA and then incubated with antibodies, including mouse anti-SAG1 (1:500), and Alexa Fluor 488 goat anti-mouse IgG (H + L) (1:500). The number of tachyzoites in at least 200 parasitophorous vacuoles (PVs) was counted.

2.10 Egress assay

To investigate whether SR2 is involved in the egress of *T. gondii*, 10^5 freshly egressed tachyzoites were added to HFF monolayers for 1 h at 37°C and 5% CO_2, and the un-attached tachyzoites were washed with DMEM. The samples were incubated for another 36 h and then treated with 3 μM calcium ionophore A23187 (Sigma, Burlington, MA, United States) for 2 min. The infected cells were fixed with 4% PFA and the proportion of egressed versus not egressed tachyzoites was examined microscopically to evaluate the egress efficiency as previously described (Liang et al., 2021).

2.11 Examining the role of SR2 in acute and chronic infection

Female mice (6 mice/group) were infected by intraperitoneal (i.p.) injection with 100 tachyzoites of RH, RHΔ*sr2* and RHΔ*sr2*C or 200 tachyzoites of Pru, PruΔ*sr2* and PruΔ*sr2*C to evaluate the impact of SR2 on acute and chronic *T. gondii* infection, respectively. A plaque assay was performed to ascertain the viability of the tachyzoites used in mouse infection. Mice were monitored twice a day for 30 days and euthanized once they reach the humane endpoint (loss of ≥20% of initial body weight). The brains of mice infected by Pru, PruΔ*sr2* and PruΔ*sr2*C were collected at 30 days after infection and homogenized with 1 mL PBS to count the number of brain cysts as previously

described (Zheng et al., 2023). The experiments were repeated two independent times.

2.12 Determination of antibody levels induced by RHΔ*sr2* strain

Enzyme-linked immunosorbent assay (ELISA) was used to evaluate concentration of antibodies in the serum of survived mice infected by RHΔ*sr2* strain as previously described (Wang et al., 2017). In brief, a 96-well plate (Thermo Fisher Scientific, Waltham, MA, United States) was coated overnight with soluble tachyzoite antigen (STAg), blocked with 5% BSA, and then incubated with mouse serum as primary antibodies (1:50), and secondary antibodies, including horseradish-peroxidase-conjugated goat anti-mouse IgG (1:250), anti-mouse IgG1 (1:500), and IgG2a (1:500) (Abcam, Cambridge, MA, United States). After addition of 2% sulfuric acid, the absorbance was read at 450 nm using iMark microplate Absorbance reader (Bio-Rad, Hercules, CA, United States). To assess the protective efficacy against *T. gondii* re-infection, mice that survived infection by RHΔ*sr2* were challenged, 45 days after infection, with 500 RH tachyzoites. Another group of mice was injected with 500 RH tachyzoites and served as a control. All mice were monitored twice daily.

2.13 *In vitro* bradyzoite differentiation

For bradyzoite differentiation, Pru, PruΔ*sr2* and PruΔ*sr2*C tachyzoites were induced under alkaline conditions as previously described (Wang et al., 2022). Briefly, tachyzoites were allowed to invade HFF monolayers for 4 h at 37°C with 5% CO_2, and then cultured in RPMI-HEPES medium (pH 7.8, pH 8.0, and pH 8.2) for 48 h at 37°C without CO_2. The medium was replaced every day to maintain the alkaline condition of the culture. IFA was performed to observe the proportion of parasites stained with rabbit anti-IMC1 (1:500) and Alexa Fluor 594 goat anti-rabbit IgG (H + L) (1:500) and the parasite cysts were stained with FITC-DBL.

2.14 Bioinformatic analysis of *Toxoplasma gondii* SR2

The data about *T. gondii* SR2, including amino acid sequence, signal peptide and transmembrane domain, were retrieved from ToxoDB.[1] The domains of SR2 protein were analyzed by using ExPASy Server[2] and relevant reference as previously described (Wan et al., 2019). The 3D structure of SR2 was constructed using the SWISS-MODEL server,[3] based on the crystal structure of *T. gondii* RNA recognition motif-containing protein TGP89_217540 (PDB: A0A086K082.1.A). BLAST search for homologous proteins of SR2 was performed and phylogenetic tree based on amino acid sequence of SR2 protein was constructed by maximum likelihood method using MEGA-X software.

1 http://toxodb.org
2 https://www.expasy.org
3 https://swissmodel.expasy.org

2.15 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 8.01, GraphPad Software, Inc). One-way ANOVA and two-tailed, unpaired Student *t*-test analyses were used for comparisons between three or more groups or between two groups, respectively. The experiments were repeated three independent times. The results are shown as mean ± standard deviation (SD). The level of statistically significant differences is indicated on the figures by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

3 Results

3.1 Characterization and localization of SR2

According to the ExPASy Server,[4] the predicted SR2 protein (TGME49_217540) consists of 351 amino acids and does not have a signal peptide or a transmembrane domain, but has two RRM domains at the N-terminus and one RS domain at the C-terminus (Figure 1A; Supplementary Figure S1). Based on the Swiss model, the structure of SR2 protein conformed to the classical structural characteristics of the SR protein family (Figure 1B) (Shepard and Hertel, 2009). Phylogenetic analysis showed that SR2 had high homology to the alternative splicing factor SR1 (PfSR1), which plays an essential role in the proliferation of *P. falciparum* (Figure 1C) (Eshar et al., 2012). To explore the subcellular localization of SR2, a 6 × HA epitope tag was successfully inserted into the C-terminus of SR2. IFA showed that SR2 was localized in the nucleus in the tachyzoite and bradyzoite stages (Figure 2A). WB analysis showed that cell lysates of 6 × HA tagged strain expressed the fusion protein and its size was ~52 kDa, which conforms with the predicted size of the protein (Figure 2B).

3.2 Successful disruption and complementation of SR2 in *Toxoplasma gondii* RH and Pru strains

To investigate the biological function of SR2, 5′ HR-DHFR-3′ HR homologous fragment was used to disrupt coding region of SR2 (Figure 3A). The successful deletion of SR2 gene was confirmed using PCR2, where no bands were amplified in the Δ*sr2* strains, however, ~500 bp bands were detected in the wild-type strains. The correct replacement of the 5′ HR-DHFR-3′ HR fragment was confirmed by the amplification of ~1,000–1,500 bp bands from the Δ*sr2* strains by PCR1 and PCR3. These results showed that SR2 was successfully knocked out in type I RH strain and type II Pru strain (Figure 3C). Δ*sr2* complemented strains were constructed to further elucidate the function of SR2 (Figure 3B). PCR and WB analyses revealed that bands were amplified in the Δ*sr2*C strains, however these fragments were absent in the wild-type and Δ*sr2* strains (Figures 3D,E). Successful complementation of the coding region of SR2 fused with 3 × HA tag to Δ*sr2* strains was verified by IFA (Figure 3F).

4 https://www.expasy.org

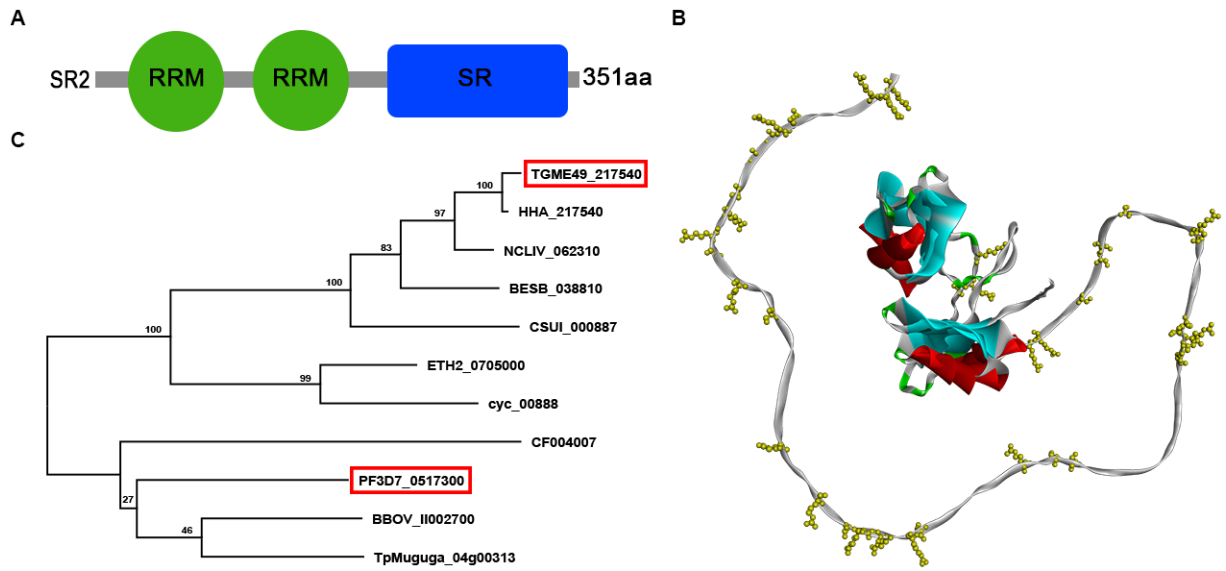

FIGURE 1
Characterization of the splicing factor SR2 in *Toxoplasma gondii*. **(A)** Domain analysis of splicing factor SR2 using ExPASy server. SR2 protein has two RRM domains (Green box) at the N-terminus and one RS domain (blue box) at the C-terminus. **(B)** Schematic representation of the 3D structure of the SR2 protein using SWISS-MODEL server based on the crystal structure of *T. gondii* RNA recognition motif-containing protein TGP89_217540 (PDB: A0A086K082.1.A). **(C)** Based on the amino acid sequence of SR2, phylogenetic tree of SR2 protein was constructed by maximum likelihood method using MEGA-X software.

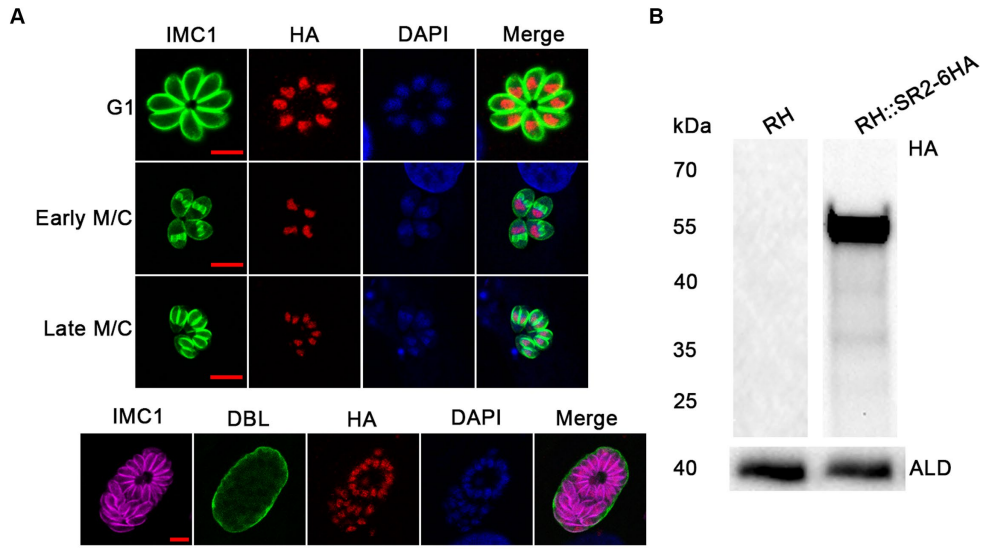

FIGURE 2
Subcellular localization and expression of SR2 in *T. gondii*. **(A)** Subcellular localization of SR2 in tachyzoites and bradyzoites. Rabbit anti-IMC1, mouse anti-HA and DAPI were used to stain the inner membrane complex (green), localize the tagged protein (red) and stain the nucleus (blue), respectively. Scale bars, 5 μm. IFA showed that SR2 in tachyzoites and bradyzoites was localized to the nucleus. **(B)** Western blotting confirmed the correct expression of SR2 coding region fused with 6HA tag as evidence by detection of a ~ 52 kDa band. Anti-aldolase (ALD) was regarded as a loading control.

3.3 Deletion of SR2 is dispensable for the growth or infectivity of *Toxoplasma gondii*

To assess the growth kinetics of Δ*sr2* strains, plaque assay was performed. Seven or nine days after infection of HFFs, there were no significant differences in the size and number of plaques between the wild-type, the Δ*sr2* and the complemented strains (Figures 4A,E), indicating that absence of SR2 does not affect the lytic cycle of tachyzoites *in vitro*. Next, we performed the invasion, replication, and egress assay. For invasion assay, HFF monolayers were infected by RH, RHΔ*sr2* and RHΔ*sr2*C strains and the number of extracellular and intracellular tachyzoites was determined using fluorescence

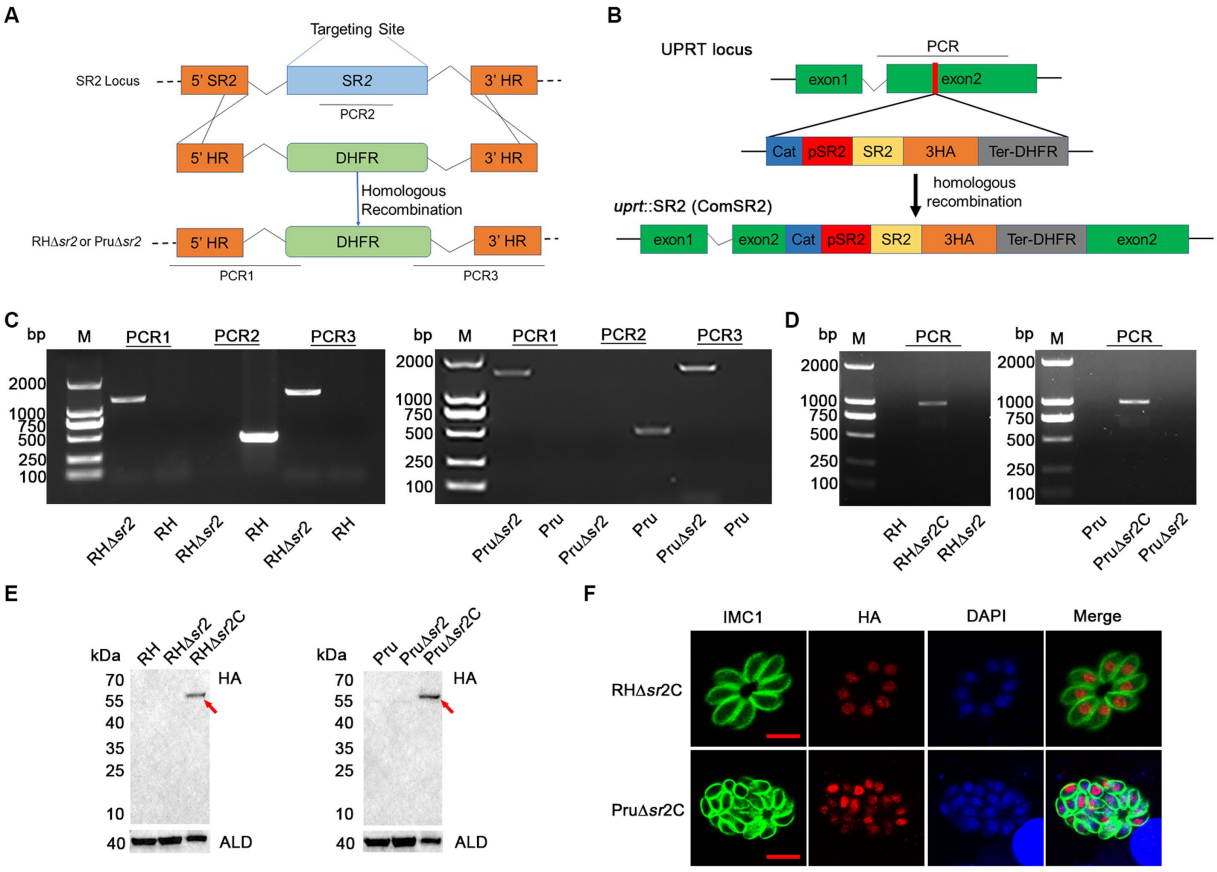

FIGURE 3
Generation of Δ*sr2* and Δ*sr2*C in type I RH strain and type II Pru strain. **(A)** Schematic diagram of CRISPR-Cas9 mediated homologous recombination. **(B)** Schematic representation of the insertion of coding region of SR2 fused with 3 × HA tag driven by SR2 promoter at the UPRT locus of Δ*sr2* strains. **(C)** Confirmation of the construction of Δ*sr2* strains by PCR. PCR2 was used to determine whether SR2 was deleted, and PCR1 and PCR3 were used to determine whether 5′ HR-DHFR-3′ HR fragment was successfully inserted into the target gene sequence. **(D-F)** Identification of Δ*sr2*C strains by PCR, IFA, and Western blotting analyses. PCR and Western blotting analyses showed that the expected fragments were amplified from Δ*sr2*C strains of type I RH and type II Pru, but not from wild-type and Δ*sr2* strains. IFA analysis showed the successful expression of the coding region of SR2 fused with 3HA tag at the UPRT locus of Δ*sr2* strains, confirming that SR2 was localized to the nucleus. Scale bars, 5 μm.

microscopy. The results showed that disruption of SR2 did not significantly affect the parasite invasion ability (Figure 4B). Intracellular replication assay was performed to evaluate proliferation efficiency of RHΔ*sr2* strain based on the number of tachyzoites in each PV, 24 h post infection. RHΔ*sr2* tachyzoites exhibited similar proliferation kinetics compared to the RH and RHΔ*sr2*C tachyzoites (Figure 4C). To investigate the egress ability of RHΔ*sr2* strain, HFF monolayers were infected for 36 h and treated with 3 μM calcium ionophore A23187. The results showed that RHΔ*sr2* had the same egress efficiency as the RH and RHΔ*sr2*C (Figure 4D). These results indicate that absence of SR2 does not cause significant alteration in the parasite's ability for invasion, replication, or exit of the host cells, suggesting that SR2 does not play an important role in the parasite growth *in vitro*.

3.4 SR2 is important in acute and chronic toxoplasmosis of mice

To investigate the *in vivo* effect of the absence of SR2 on the parasite's virulence, female mice (6 mice/group) were intraperitoneally infected by 100 tachyzoites of RH, RHΔ*sr2* and RHΔ*sr2*C or 200 tachyzoites of Pru, PruΔ*sr2* and PruΔ*sr2*C and the number of mice that exhibited humane endpoint was recorded. The results showed that disruption of SR2 in type I RH strain and type II Pru strains resulted in varying degrees of attenuated virulence (33.33% RHΔ*sr2*; 0% RH or RHΔ*sr2*C; 83.33% PruΔ*sr2*; 33.33% Pru or PruΔ*sr2*C). The complementation of SR2 gene rescued the virulence defect caused by SR2 deletion (Figures 5A,D).

To confirm that the survived mice are infected with viable RHΔ*sr2* parasites, antibody levels produced by survived mice were determined using ELISA. The levels of IgG and IgG2a in RHΔ*sr2*-infected mice were significantly higher than those in the control group (uninfected group) ($p < 0.0001$), but there were not significant changes in the IgG1 level ($p > 0.05$) (Figure 5B). The IgG2a/IgG1 ratio suggests a Th1 immune response. To investigate immune protection conferred by RHΔ*sr2* strain, survived mice were challenged with 500 RH tachyzoites 45 days after initial infection. The results showed that mice infected by RHΔ*sr2* tachyzoites survived the challenge with wild-type RH tachyzoites for 30 days, while mice in the uninfected group reached the human endpoint within nine days ($p < 0.01$), suggesting that immune

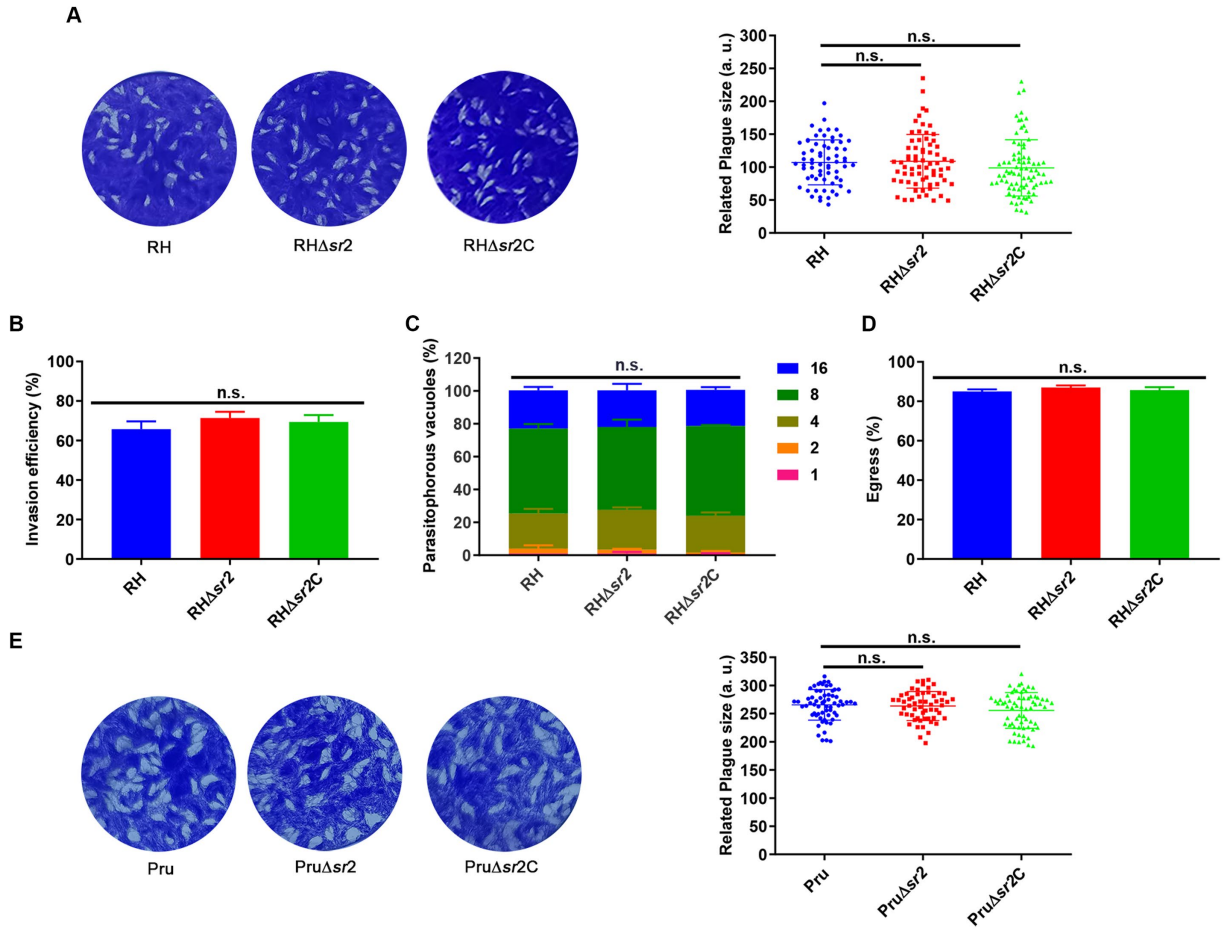

FIGURE 4
SR2 is dispensable for the growth of *T. gondii* in type I RH strain and type II Pru strain. **(A)** Representative plaques produced by RH, RHΔ*sr2* and RHΔ*sr2*C strains. No significant differences were detected in the number and size of plaques formed by RH, RHΔ*sr2* and RHΔ*sr2*C strains infecting HFFs for seven days. **(B-D)** SR2 was not essential for the invasion, intracellular replication, and egress of RH, RHΔ*sr2* and RHΔ*sr2*C tachyzoites. **(E)** Representative plaques formed by Pru, PruΔ*sr2* and PruΔ*sr2*C strains showing no significant differences in the number and size of plaques formed by Pru, PruΔ*sr2* and PruΔ*sr2*C strains nine days post-infection. n.s., not significant.

response produced by the survived mice was effective in protecting against virulent infection (Figure 5C).

To investigate the role of SR2 in chronic infection, the number of brain cysts was quantified in mice infected by 200 tachyzoites of Pru, PruΔ*sr2* and PruΔ*sr2*C 30 days after infection. The results revealed significant reduction of ~80% in the brain cyst burden in mice infected by PruΔ*sr2* strain (46 ± 25 cysts/brain) compared to those infected by Pru (216 ± 35 cysts/brain) or PruΔ*sr2*C strains (224 ± 35 cysts/brain) ($p < 0.0001$) (Figure 5E), indicating that deletion of SR2 causes severe disruption of cyst formation.

3.5 Deletion of SR2 does not affect the bradyzoite differentiation *in vitro*

In view of the severe defects in cyst formation caused by disruption of SR2 *in vivo*, Pru, PruΔ*sr2* and PruΔ*sr2*C strains were exposed to different alkaline conditions (pH 7.8, pH 8.0, and pH 8.2) to study whether SR2 affects tachyzoite to bradyzoite transformation *in vitro*. The tachyzoite to bradyzoite transformation efficiency was evaluated by counting cyst wall stained with FITC-DBL using fluorescence microscopy. Surprisingly, the results showed that tachyzoite to bradyzoite conversion efficiency of PruΔ*sr2 in vitro* was not significantly different under different alkaline conditions compared with Pru or PruΔ*sr2*C strains, indicating that SR2 does not contribute to the tachyzoite to bradyzoite conversion *in vitro* (Figures 6A,B).

4 Discussion

The success of *T. gondii* lies in its remarkable ability to respond and adapt to various ecological and physiological niches, which involves orchestrating the transcription of many genes. As key regulators of AS, SR proteins are involved in key processes such as splicing, mRNA export, mRNA stability, and translation (Huang and Steitz, 2005). The multi-faceted roles of SR proteins and the fact that disruption of *P. falciparum* SR1 and *T. gondii* SR3 results in significant growth defect of these parasites (Eshar et al., 2012; Yeoh et al., 2015) inspired us to investigate the function of SR2 protein in *T. gondii*.

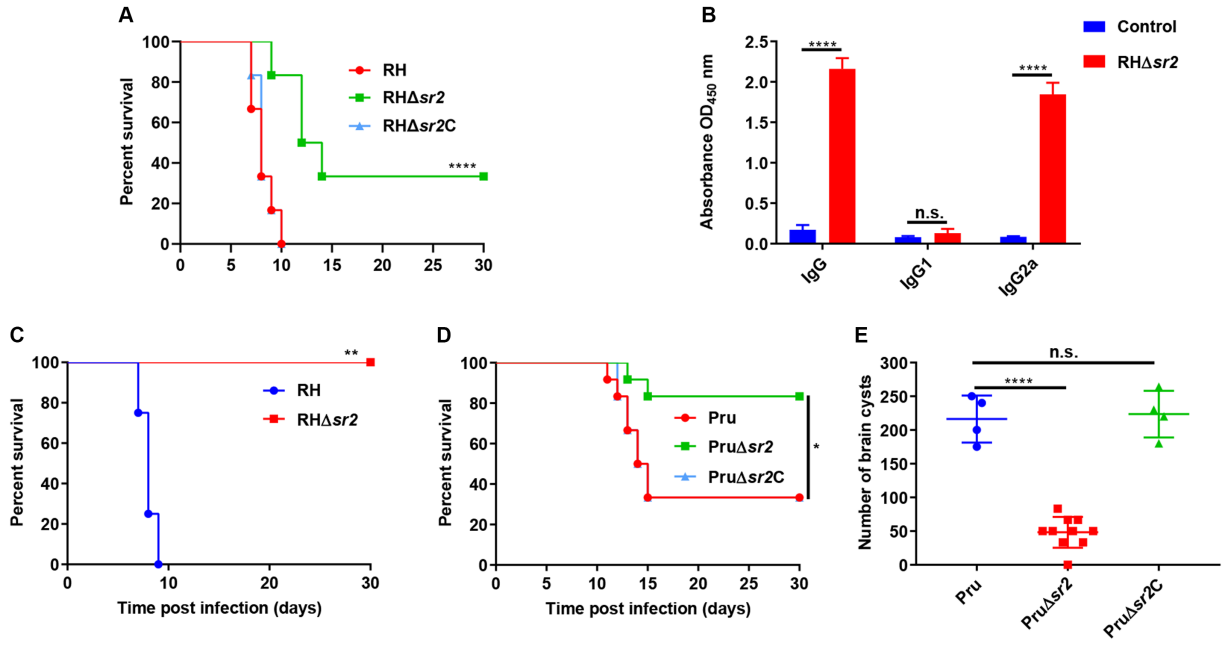

FIGURE 5
SR2 is important for *T. gondii* virulence. **(A)** Probability of survival of mice (6 mice/group) injected intraperitoneally (i.p.) with 100 tachyzoites of RH, RHΔ*sr2* and RHΔ*sr2*C. **(B)** Detection of high IgG and IgG2a antibody levels showed that the survived mice infected by viable RHΔ*sr2* tachyzoites induced Th1 immune response. **(C)** Probability of survival of RHΔ*sr2*-infected mice i.p. challenged, 45 days after infection, with 500 RH tachyzoites. **(D)** Survival of mice (6 mice/group) i.p. infected by 200 tachyzoites of Pru, PruΔ*sr2* and PruΔ*sr2*C. **(E)** The burden of brain cysts detected in mice infected by 200 tachyzoites of Pru, PruΔ*sr2* and PruΔ*sr2*C was determined at 30 days after infection. n.s., not significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, compared with control.

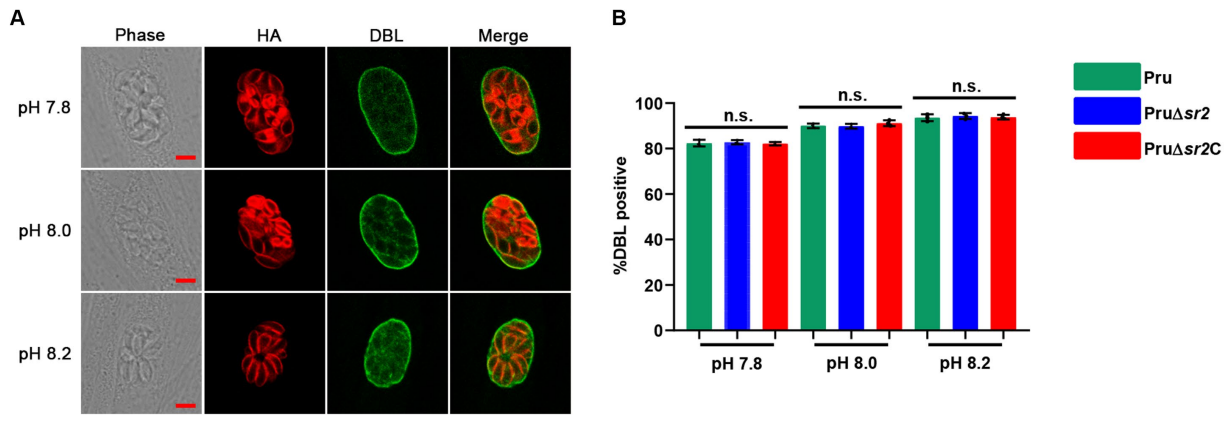

FIGURE 6
Deletion of SR2 does not affect tachyzoite to bradyzoite differentiation *in vitro*. **(A)** Pru, PruΔ*sr2* and PruΔ*sr2*C tachyzoites were allowed to invade HFFs for 4 h under normal culture conditions and then incubated in the absence of CO_2 and under different alkaline stress conditions (pH 7.8, 8.0 and 8.2) for additional 48 h. Rabbit anti-IMC1 and FITC-DBL were used to stain the parasites (red) and cyst wall of bradyzoite (green), respectively. Scale bars, 5 μm. **(B)** Tachyzoite to bradyzoite conversion efficiency of Pru, PruΔ*sr2* and PruΔ*sr2*C under different alkaline conditions. No significant differences were detected in the proportion of DBL positive vacuoles of Pru, PruΔ*sr2* and PruΔ*sr2*C.

SR2 belongs to a family of multi-domain RNA binding proteins that regulate alternative splicing of genes involved in many biological processes (Huang and Steitz, 2005). SR proteins harbor structural modules for RNA recognition at the amino terminus, such as RRM domains and a RS domain at the carboxyl terminus required for protein–protein interaction (Shepard and Hertel, 2009). SR proteins are located in the nucleus and mainly localize to a subnuclear region in a process mediated by a nuclear localization signal, mainly the RS domain (Cáceres et al., 1997). To investigate the subcellular localization of SR2 in *T. gondii*, a 6×HA-tag was inserted at the C-terminus of SR2 in RH strain and IFA analysis showed that SR2 was localized to the parasite nucleus in tachyzoites and bradyzoites, suggesting that SR2 is a SR protein.

Next, we investigated the role of the splicing factor SR2 in the growth and pathogenicity of *T. gondii*. *T. gondii* proliferation is based on successive lytic cycles involving the parasite invasion, intracellular proliferation, and egress of the host cell (Hortua Triana et al., 2018; Sanchez and Besteiro, 2021). Thus, we examined the effect of deletion

of SR2 in type I RH strain and type II Pru strain on the parasite ability to invade, replicate and exit the host cells. Consistent with a previous report on the CRISPR-Cas9 phenotypic value of SR2 (Sidik et al., 2016), disruption of SR2 in type I RH strain and type II Pru strain did not significantly affect the parasite growth (based on the size and number of plaques produced by the parasite), invasion ability, intracellular replication efficiency, and egress ability.

Splicing factors have been shown to mediate the virulence of various pathogens. For example, KH-QUA2 (84KQ) motifs at the C-terminus of *Entamoeba histolytica* U2AF2 play roles in splicing of several virulence genes, and absence of 84KQ motifs alters splicing and reduces the parasite virulence (González-Blanco et al., 2022). The deletion of *Ustilago maydis Rrm75*, which encodes three RRM domains and glycine-rich repeats, fails to produce splicing variants at the 3′-alternative splicing locus of the third exon through AS, resulting in abnormal growth and virulence phenotypes (Rodríguez-Kessler et al., 2012). Therefore, we hypothesized that deletion of SR2 may influence the pathogenicity of *T. gondii*. We explored the role of splicing factor SR2 in the virulence of *T. gondii* during acute and chronic infection in mice. The results showed that the virulence of type I RH strain and type II Pru strain lacking splicing factor SR2 resulted in varying degrees of attenuation. The different attenuated virulence may be attributed to the distinct inherent differences in the virulence between type I RH strain and type II Pru strain (Howe and Sibley, 1995). These results show the adverse effect of SR2 deletion on the parasite virulence, which may be caused by dysregulated splicing. The mechanism by which SR2 affects the virulence of *T. gondii* remains to be investigated.

Notably, we observed a significant reduction of brain cyst burden in mice infected by Pru*Δsr2* compared with mice infected by Pru or Pru*Δsr2*C. However, the same strains did not exhibit significant differences in the tachyzoite to bradyzoite differentiation rate *in vitro*. This paradox could suggest the presence of host immune or molecular factors that contribute to reduction in tachyzoite to bradyzoite conversion in mice, but are lacking in the cell culture (Li D. et al., 2023; Li T. T. et al., 2023). However, the nature of this difference and the gene regulatory mechanisms controlling temporal expression of SR2 under *in vitro* and *in vivo* conditions remain to be elucidated.

The humoral immune response of mice infected by attenuated parasite strains can provide protection against re-infection by *T. gondii* (Zhang et al., 2018; Li D. et al., 2023; Li T. T. et al., 2023). Anti-*T. gondii* specific IgG2a and IgG1 isotypes levels are used as indicators for the balance between the Th1 and Th2 responses (Wang et al., 2017, 2018). Our results showed that mice which survived acute infection developed high level of IgG and IgG2a but low IgG1 level, consistent with Th1 immune response which is essential for protection against *T. gondii* re-infection (Rostamian et al., 2017; Wang et al., 2017). These results show that despite the low level of IgG1 antibodies, mice vaccinated by RHΔsr2 still have protective immunity against *T. gondii*, suggesting that IgG2a isotype correlated with increased protection against lethal *T. gondii* challenge than the IgG1 isotype, in agreement with previous studies (Wang et al., 2017, 2018).

5 Conclusion

We investigated the role of the splicing factor SR2 in the pathogenicity of *T. gondii*. Although disruption of SR2 did not significantly affect *T. gondii* growth and tachyzoite to bradyzoite differentiation *in vitro,* it markedly impaired the parasite virulence and cyst formation *in vivo*. These results suggest that SR2 deletion may affects the parasite virulence by disrupting the splicing regulatory network in *T. gondii*. Further investigations are required to elucidate the gene regulatory network controlling the expression and biological functions of SR2. Given the current situation of limited therapeutic options against *T. gondii* infection, searching for more effective drugs is needed to improve clinical anti-*T. gondii* chemotherapeutics. In this regard, screening and testing functional inhibitors against *T. gondii* SR2 is worthwhile.

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 authors.

Ethics statement

The animal study was approved by the Animal Research Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

X-JW: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. JG: Formal analysis, Investigation, Methodology, Validation, Writing – original draft. X-NZ: Formal analysis, Investigation, Methodology, Writing – review & editing. HE: Conceptualization, Resources, Visualization, Writing – review & editing. T-TL: Investigation, Methodology, Writing – review & editing. Y-JK: Investigation, Writing – original draft. MW: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. X-QZ: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, 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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1302512/full#supplementary-material

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Clinical and microbiological features of host-bacterial interplay in chronic venous ulcers *versus* other types of chronic skin ulcers

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Introduction: Chronic venous ulcers of the lower limbs develop in the context of advanced venous disease and have a significant impact on the patient's quality of life, being associated with depression and worrisome suicide rates, as well as with an economic burden caused by increased medical care costs and high epidemiological risks of healthcare associated infections and emergence of strains resistant to multiple classes of antibiotics and/ or antiseptics. Although numerous studies have investigated the composition of the chronic wounds microbiome, either by culture-dependent or independent methods, there are no data on the association between virulence and resistance profiles of strains isolated from venous ulcers and the clinical picture of this pathology. The elucidation of pathogenic mechanisms, at both phenotypic and molecular level, is crucial in the fight against these important human microbial agents, in order to develop novel biomarkers and discover new therapeutic targets.

Methods: In this study we aimed to characterize the phenotypic virulence profiles (including the ability to develop biofilms) of microorganisms isolated from chronic skin wounds and to correlate them with the clinical symptomatology. Considering the high incidence of *Staphylococcus aureus* infections in chronic ulcers, but also the ability of this species to develop multi-drug resistance, we performed an more in-depth study of the phenotypic and genotypic virulence profiles of methicillin-resistant *Staphylococcus*.

Results: The study revealed important differences regarding the clinical evolution and virulence profiles of microorganisms isolated from lower limb wounds, as well as between patients diagnosed with chronic venous ulcers and those with lesions of different etiology.

KEYWORDS

chronic wound, host-microbiome, bacterial virulence factors, methicillin resistant *Staphylococcus aureus* (MRSA), tolerance at antimicrobials of biofilm cells, bio-marker

1 Introduction

Most chronic wounds of the lower limbs are of venous etiology (70%–90%; Kahle et al., 2011; Stanek et al., 2023). Chronic venous ulcers of the lower limbs are defined as wounds that develop in the context of advanced venous disease, with a slow healing rate and no tendency to heal over 3 months despite adequate treatment or not fully healed at 12 months (Kahle et al., 2011; Stanek et al., 2023). They have a significant impact on the patient's quality of life, being associated with depression and worrisome suicide rates, as well as an economic burden due to increased medical care costs (dressings, hospitalization, and others; Kahle et al., 2011; Weller et al., 2021; Stanek et al., 2023). Moreover, they imply an epidemiologic risk. In the context of recurrent hospitalizations and long-term antibiotic treatment, a worrisome issue arises both in Romania, as well as at global level: healthcare associated infections and the emergence of strains resistant to multiple classes of antibiotics and/ or antiseptics (Rhoads et al., 2012; Salavastru et al., 2012; Mihai et al., 2014), with medical and socio-economic disastrous consequences. Therapeutic approaches are limited, having an important impact on the chances of healing and/or survival.

Although the exact pathogenicity of delayed healing in chronic venous ulcers is unknown, several factors have been described: endogenous (e.g., vascular disease, comorbidities, immunity of the host, the presence of vicious scars after local trauma or surgery) or exogenous (e.g., bacterial colonization or infection, local therapeutic agents that impair healing, excessive hygiene; Cooper et al., 2014; Percival et al., 2014; Weller et al., 2021). Marques et al. (2023) reviewed the prognostic factors associated with delayed healing of wounds in adults: male gender, sedentarism with the decline in activities of daily life, history of ulcers, comorbidities such as renal disease, diabetes, and peripheral arterial disease, wound characteristics such as duration, area affected, and location, and wound complications such as high-stage WIfI (Wound, Ischemia, foot Infection) classification, gangrene, infection, and low ankle brachial index.

According to the International Wound Infection Institute Wound Infection Continuum (IWII-WIC) there are different, progressively more severe phases of microbial presence in a wound, ranging from contamination to critical colonization, local infection, spreading infection (cellulitis) defined by inflammation or erythema greater than two centimeters from the wound edge, and systemic infection/sepsis (International Wound Infection Institute (IWII), 2022). A local infection is a stage when wound bacteria proliferate and activate host immune defense mechanisms, leading to local tissue damage and impaired healing [International Wound Infection Institute (IWII), 2022]. Local infection causes changes within the wound (hypergranulation and/or friable granulation, bleeding, epithelial bridging and pocketing, increased exudate, purulent discharge, delayed wound healing beyond expectations, new or increasing pain, and/or increasing malodor) and its immediate skin surroundings [erythema, local warmth, swelling, pain; International Wound Infection Institute (IWII), 2022].

Every open wound has bacteria colonizing it, however not every contaminated wound gets infected. The main factors that influence the development of wound infection are represented by: i) the immune status of the host and, implicitly, the capacity to combat opportunistic pathogens; ii) the wounds' bacterial load, since more microorganisms have a better chance to counteract the host's immune defense mechanisms; iii) the contaminant bacterial strains, since some are more virulent, including a higher ability to develop biofilms; iv) the polymicrobial associations, since some microorganisms appear to develop synergistic effects [social microbiology; International Wound Infection Institute (IWII), 2022]. Patients suffering from systemic or local immunosuppression (e.g., advanced *Human immunodeficiency virus* infection, the use of medication in organ recipient patients, the topical preoperative corticosteroid treatment, others) are more susceptible to delayed wound-healing and wound-related complications issues (Alqatawni et al., 2020; Varga et al., 2021).

Chronic wound bacteria impede normal healing by several mechanisms: microbial invasion, the development of virulence traits that increase the pathogenicity of bacteria (e.g., production of soluble virulence factors, the development of mono- or polymicrobial biofilms), resistance and/ or tolerance to antimicrobial agents, and to host immune defense mechanisms. Moreover, bacterial synergism in polymicrobial wound infections (e.g., *Staphylococccus aureus*, *S. aureus* and *Pseudomonas aeruginosa*, *P. aeruginosa*) promotes the virulence and persistence of infection, as well as a decreased response to therapy (DeLeon et al., 2014).

The virulence of bacteria is a context dependent, multifactorial, and dynamic property (Diard and Hardt, 2017). There are very few studies that have correlated the virulence profile of bacteria isolated from different types of chronic infections with the host immune response or with the clinical outcome. Sotto et al. (2008) studied the virulence potential of 132 *S. aureus* strains isolated from diabetic foot ulcers. They showed that a particular combination of virulence genes (*cap8*, *sea*, *sei*, *lukE*, and *hlgv*) was statistically correlated with infection and may serve as a prediction factor for the wound status at the follow-up (Sotto et al., 2008). Moreover, Wu et al. (2023) demonstrated that the levels of glucose and its metabolites impact on the virulence and inflammatory response of *S. aureus* strains isolated from diabetic foot ulcers. Regarding chronic venous ulcers, Cwajda-Białasik et al., (2021) proved that culture-positive lesions, and, therefore, colonization or infection status, are more frequent in older patients (age > 65 years) with chronic and larger wounds (ulcer duration > 12 months, ulceration area > 8.25 cm^2). Different from the studies of diabetic foot ulcers, Gajda et al. (2021) did not observe a significant correlation between the virulence of *S. aureus* strains isolated from venous ulcers and the presence of certain genes, in particular, between *pvl* genes and the *spa* type.

Staphylococcus aureus is the most frequent species isolated from chronic wounds and it is well known that methicillin-resistant *S. aureus* (MRSA) causes severe infections. The strains persist in the hospital environment, where, under the selective pressure of antibiotics, they evolve, with the expression of β-lactamase coding genes, as well as some metabolic and virulence genes. Moreover, they can transfer resistance genes to other species directly or indirectly. Besides antibiotic resistance, *S. aureus* triggers disease through various pathogenic mechanisms, involving both exotoxins, including pore-forming toxins, staphylococcal superantigens, as well as bacterial adhesins and biofilm development (Baba et al., 2002).

Although numerous studies have investigated the composition of the microflora of chronic wounds, either by classical diagnostic methods or by combining molecular methods, there are few comparative data on the virulence and resistance profiles of strains isolated from venous ulcers with the manifested clinical picture. The elucidation of pathogenic mechanisms, at the molecular level, is

crucial in the fight against these important human microbial agents, in order to discover new therapeutic targets.

In this study we aimed to characterize the phenotypic virulence profiles (including the ability to develop biofilms) of microorganisms isolated from chronic skin wounds (venous, arterial and mixed arterio-venous ulcers, pressure sores, wounds secondary to surgery or associated with abscesses, paraneoplastic ulcerations or secondary to autoimmune vesiculo-bullous diseases—pemphigus vulgaris, bullous pemphigoid) and to correlate the data obtained with the clinical picture. Considering the high incidence of *S. aureus* infections at the level of chronic ulcers, but also the problem of developing multi-drug resistance, we also performed an in-depth study of this bacterial species, by analyzing the phenotypic and genotypic virulence profile of MRSA strains.

2 Materials and methods

We performed an observational, prospective study that included 80 patients diagnosed with chronic skin wounds (39 venous ulcers, 41 other types of chronic skin wounds), showing clinical signs of infection, hospitalized in the "Elias" Emergency University Hospital, Bucharest, Romania, during the last 5 years. Other types of chronic skin wounds were: arterial ulcers (Cooper et al., 2014), mixed arterio-venous ulcers (Rhoads et al., 2012), pressure sores (Mihai et al., 2014), wounds secondary to surgery (Weller et al., 2021) or associated with abscesses (International Wound Infection Institute (IWII), 2022), paraneoplastic ulcerations (Kahle et al., 2011) or secondary to autoimmune vesiculo-bullous diseases—pemphigus vulgaris, bullous pemphigoid (Marques et al., 2023). There were excluded patients who received systemic antibiotic treatment during the past month or topical antimicrobials during the past week before hospital admission, who had a diagnosis of immunosuppression or under treatment with immunosuppressive drugs/medication. Also, there were excluded the cases where there were lacking clinical criteria of wound infection (Bianchi et al., 2016; International Wound Infection Institute (IWII), 2022).

Monitoring of patients began on the date of the diagnosis of a chronic, superinfected wound. The follow-up was performed for all patients at 1 month and at 6 months after diagnosis, analyzing the clinical evolution, including the status of a healed wound (complete epithelialization). The follow-up was objectified by the RESVECH 1.0 scale and by non-invasive imaging through serial comparative photography. Patients with more frequent hospitalizations were evaluated each time. The follow-up period ended 6 months after diagnosis, in the case of skin healing, or 1 year in patients with persistent skin lesions.

An informed consent was obtained from each patient. The study protocol is in accordance to the ethical prerogatives of the 1975 Declaration of Helsinki, respecting the Good Clinical Practice (GCP) hospital admission, standards to the same extent, and was approved by the Ethics Commission of the "Elias" University Emergency Hospital, Bucharest.

The laboratory tests were performed at the "Elias" Emergency University Hospital, the Institute of Research of the University of Bucharest, Romania and the "Cantacuzino" National Medico-Military Institute for Research and Development. The identity of the bacterial strains was confirmed using classical microbiological phenotypic methods, automated Vitek 2 system and Phoenix BD system. Several phenotypic and genotypic virulence and antibiotic resistance features of the bacterial strains were evaluated.

2.1 Characteristics of the host

Clinical data was collected by anamnesis (the etiology, location and the presence of clinical signs of infection for each chronic wound, the patient's history of associated aggravating pathologies), full body examination, and wound assessment. In order to establish a positive diagnosis, studies of arterial and venous supply were performed in patients with chronic wounds of the lower limbs (ultrasound examination, ankle-brachial index). Chronic venous disease was classified according to the CEAP classification and the Venous Clinical Severity Score (Lurie et al., 2020).

If there was a clinical suspicion of neoplasm, inflammatory or autoimmune disease (e.g., vesiculo-bullous disorder) or if the skin ulcers were refractory to 3 months or more of appropriate treatment, a wound biopsy sample was taken, followed by histopathologic examination and/ or direct immunofluorescence assessment (Senet et al., 2012; Isoherranen et al., 2019; Erfurt-Berge et al., 2023).

Wound severity and progression were objectively assessed by serial imaging and by the application of RESVECH 1.0 clinical scale (Resultados Esperados de la Valoración y Evolución de la Cicatrización de las Heridas Crónicas 1.0; minimum value 0, maximum value 40, Restrepo-Medrano, 2011; Cruz et al., 2023). Therefore, each wound was evaluated according seven main items: surface, depth and affected tissues, condition of the edges of the lesion, type of tissue in the wound bed, type of exudate, signs of infection/inflammation (increasing pain, perilesional erythema, perilesional edema, high temperature, increasing exudate, purulent exudate, friable tissue or tissue that bleeds easily, stagnant wound non-progressive tissue, biofilm-compatible tissue, odor, hyper-granulation, increased wound size, satellite lesions, tissue pallor), and incidence of pain (Restrepo-Medrano, 2011; Cruz et al., 2023).

Laboratory markers of inflammation and/or infection were also assessed (complete blood count, erythrocyte sedimentation rate, C-reactive protein, fibrinogen).

2.2 Microbiological characterization

2.2.1 Strain identification

After wound cleaning with physiological serum and debridement, samples for bacterial culture were obtained by rotating a sterile swab over a 1-cm^2 region of the wound, with pressure to express exudate, following the Levine approach. This method was also recommended by the IWII-WIC consensus—"Wound Infection in Clinical Practice" [International Wound Infection Institute (IWII), 2022], since it allows the sampling of a higher concentration of bacteria both from the wound's surface, as well as from its depth (Angel et al., 2011).

Wound swabs were tested using routine aerobic culture techniques. Specimens were Gram stained. Pure colonies were obtained by inoculating each swab onto 5% sheep blood agar, Chocolate agar, MacConkey agar (without crystal violet) and Sabouraud agar with chloramphenicol (Oxoid). All plates were incubated aerobically at 37°C for 18–24 h, with the exception of

Sabouraud plates-incubated simultaneously at 30°C and 37°C for 24–72 h. The biochemical identification was performed using the automated Vitek 2 system (bioMérieux) and Phoenix BD (Bekton–Dickinson). Commensal bacteria (coagulase-negative staphylococci) were excluded from the study. The susceptibility to antibiotics was assessed using disk diffusion method according to the CLSI guideline (*Clinical and Laboratory Standards Institute*) and automated systems (Vitek2C/Phoenix BD). D-tests were also performed to evaluate the inducible resistance to clindamycin, following the manufacturer's recommendations (Magiorakos et al., 2012). Subsequently strains were maintained at 4°C in the Microbial Culture Collection of Microbiology Laboratory, Faculty of Biology, Bucharest. For further experiments, strains were streaked on nutrient agar and incubated over night at 37°C.

2.2.2 Phenotypic assessment of the virulence profile of microorganisms

2.2.2.1 Phenotypic assessment of the adherence to human cell substrate

Bacterial adherence to HeLa and endothelial cells was performed by the adapted Cravioto's method (Cravioto et al., 1979; Lazăr, 2003; Bleotu et al., 2010; Holban et al., 2013; Curutiu et al., 2014).

Briefly, the HeLa or endothelial diploid cell monolayers were washed three times with phosphate buffered saline (PBS) and then, 1 mL of fresh medium without antibiotics was added to each well. Each well was then seeded with 1 ml of bacterial suspension of each strain prepared from bacterial mid-logarithmic phase cultures grown in nutrient broth and adjusted to 10^7 CFU/mL. The inoculated plates were incubated for 2 h at 37°C. The adherence patterns were defined as: localized adherence (LA), when individual microbial cell clusters were observed on the surface of HeLa/endothelial cells; localized aggregative adherence (LAA), when the localized aggregates showed a layered adherence pattern, like "bricks in a wall"; diffuse adherence (DA), when the bacteria adhered diffusely, covering the entire HeLa/endothelial cell surface; diffuse aggregative adherence (DAA), when the bacteria adhered diffusely, covering the entire cellular surface, having a layered adhesion pattern, like "bricks in a wall" (Figure 1; Cravioto et al., 1979; Lazăr, 2003; Bleotu et al., 2010; Holban et al., 2013; Curutiu et al., 2014; Mihai et al., 2014). For each strain, it was established an adherence index defined by the ratio between the number of HeLa/endothelial cells to which microorganisms adhered and 100 eukaryotic cells counted on the microscopic field (Cravioto et al., 1979; Lazăr, 2003; Bleotu et al., 2010; Holban et al., 2013; Curutiu et al., 2014; Mihai et al., 2014).

2.2.2.2 Phenotypic assessment of biofilm development *in vitro*

The ability of the isolated strains to produce biofilm was evaluated using the violet crystal microtiter method, with the spectrophotometric quantification of the biomass adhered to the inert substrate of 96-well plates. Overnight bacterial cultures were diluted in Tryptic Soy Broth, up to a turbidity of 0.5 McFarland (approximately 1×10^8 CFU/mL) and 20 µL of the obtained suspension were seeded in 96 multi-well plates in a volume of 180 µL liquid medium in triplicate. To allow biofilm formation, the inoculated plates were incubated for 24, 48, and 72 h at 37°C. After each incubation period, the biofilms were gently washed with PBS with the aim of removing planktonic cells. The adhered biofilm mass was treated with cold methanol for 5 min, dried at room temperature dried at room temperature, stained with stained with 0.1% crystal violet solution for 15 min, resuspended in 33% acetic acid, and the absorbance spectrophotometrically read at 492 nm. The quantity of adhered biomass is proportional to the absorbance value (Coffey and Anderson, 2014; Mihai et al., 2014; Preda et al., 2021).

2.2.2.3 Phenotypic assessment of the production of soluble virulence factors

The bacterial virulence phenotype was assessed by performing enzymatic tests for the expression of eight soluble factors, using the following specific media: 5% sheep blood agar (for alpha and beta hemolysins), 2.5% yolk agar (lecithinase test), 1% Tween 80 agar (lipase test), 15% casein agar (caseinase test), 1% gelatin agar (gelatinase), 10% starch agar (amylase), DNA agar (DN-ase test), 1% esculin iron salts (esculinase test) (Cravioto et al., 1979; Bleotu et al., 2010; Holban et al., 2013; Curutiu et al., 2014; Preda et al., 2021). Nutritive agar base was supplemented with various substrata and biochemical indicators to allow for the detection of particular bacterial enzymes. From the 24 h bacterial culture a bacterial inoculum of McFarland 0.5 (1.5×10^8 CFU/mL) was prepared and was spot inoculated with a 10 µL sterile loop in Petri dishes with specific media (Cravioto et al., 1979; Bleotu et al., 2010; Holban et al., 2013; Curutiu et al., 2014; Preda et al., 2021). The strains were incubated for 24 h at 37°C and at 25°C for the next 48 h to allow the production and observation of specific enzymatic virulence factors, evaluated after 24, 48, and 72h of incubation (Cravioto et al., 1979; Bleotu et al., 2010; Holban et al., 2013; Curutiu et al., 2014; Preda et al., 2021). The results were objectified by observing the change of culture medium aspect following the enzymatic reaction: for alpha and beta hemolysins test, the haemolytic zone surrounding the inoculation spot; for the lecithinase, lipase, caseinase, and gelatinase production, the opaque/ precipitation zone surrounding the culture spot; for DN-ase reaction, the change of color from light green to pink in the area surrounding the spot culture; for the esculinase test, the black precipitation zone surrounding the culture spot; amylase production was noted after adding iodine solution (Cravioto et al., 1979; Bleotu et al., 2010; Holban et al., 2013; Curutiu et al., 2014; Preda et al., 2021). The virulence factors production was evaluated with a score from 0 to 4 (0 was the minimum, while 4 was the maximum), depending on the diameter of the culture medium area of change around the culture spot (as exemplified in Figures 2, 3; Cravioto et al., 1979; Bleotu et al., 2010; Holban et al., 2013; Curutiu et al., 2014; Preda et al., 2021).

2.2.3 Genotypic assessment of virulence genes of MRSA strains by polymerase chain reaction

The genotypic virulence profiles were assessed by PCR for 24 MRSA strains. The molecular reactions were performed using the Corbet Thermal Cycler. Amplification products for each PCR reaction (simplex or multiplex) were visualized by electrophoresis in 1% agarose gel, stained with SYBR Safe DNS (Thermo Scientific, Bucharest, Romania) and identified based on characteristic sizes using specific molecular weight markers (M-Bench Top 100 bp DNA Ladder, Promega, United States; Preda et al., 2021).

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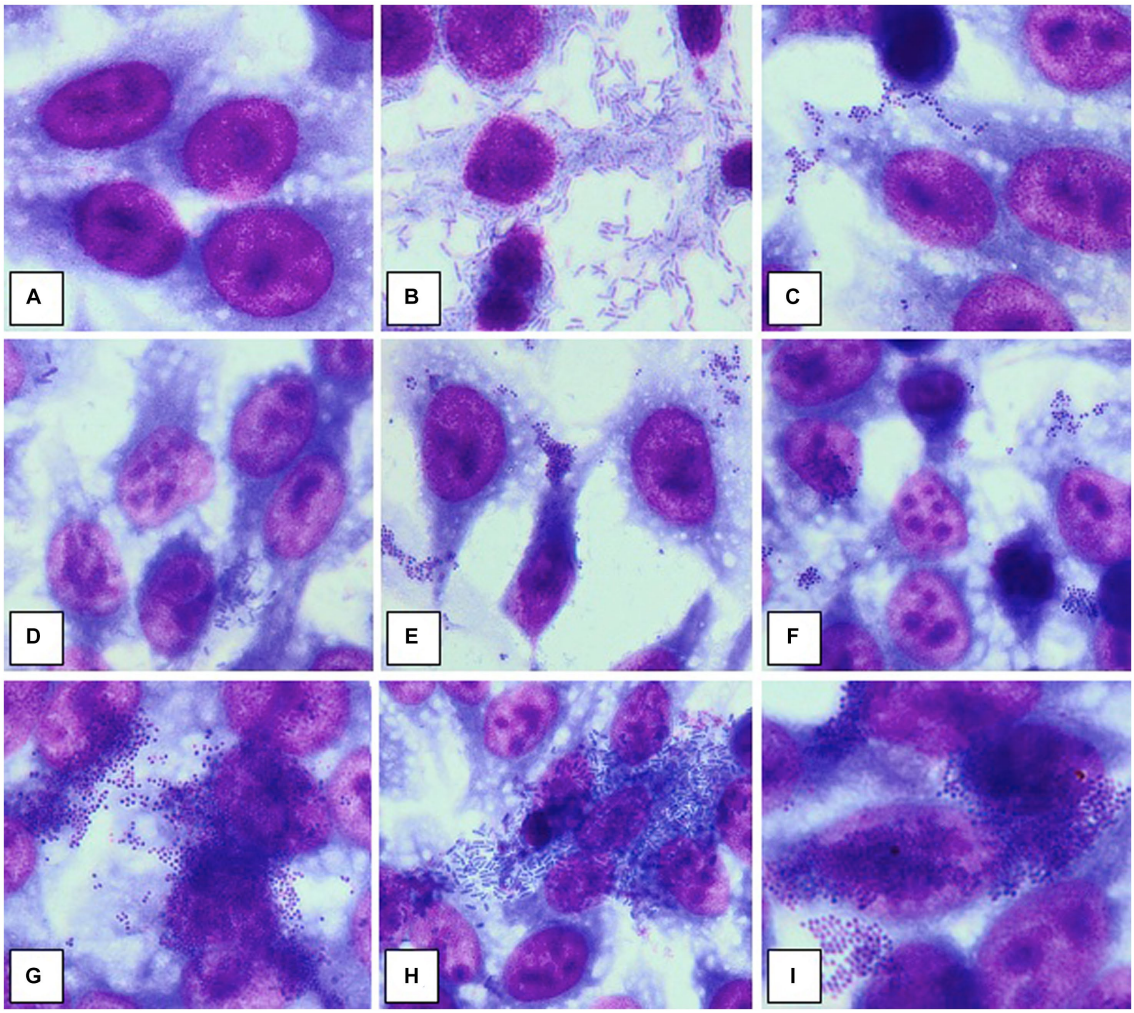

FIGURE 1
Adherence patterns to HeLa cell substrate. **(A)** No adherence; **(B)** Diffuse aggregative adherence in a strain of *Pseudomonas aeruginosa*; **(C)** Diffuse adherence in a strain of *Staphylococcus aureus*; **(D)** Aggregative localized adherence, in a strain of *Escherichia coli*; **(E)** Aggregative localized adherence, in a strain of *Staphylococcus aureus*; **(F)** Aggregative localized adherence, in a strain of methicillin-resistant *Staphylococcus aureus*; **(G)** Aggregative diffuse adherence, in a strain of methicillin-resistant *Staphylococcus aureus*; **(H)** Aggregative diffuse adherence, in a strain of *Klebsiella pneumoniae*; **(I)** Aggregative diffuse adherence, in a strain of methicillin-resistant *Staphylococcus aureus*. Source: Mihai et al. (2014). Published with the permission of authors.

Bacterial genomic DNA was extracted by the alkaline method. 1–5 MRSA colonies were suspended in 20 µL NaOH (0.05 M) and sodium dodecyl sulfate (0.25%) solution. In the next step, amplification was performed at 95°C for 15 min, followed by the addition of 180 µL TE1x buffer solution to each Eppendorf tube and centrifugation for 3 min at 13,000 r.p.m. The purity and concentration of the obtained DNA product was checked by electrophoresis in 1.5 and 2% agarose gel, 45 min at 90 V, stained with SYBR Safe DNS (ThermoScientific, Bucharest, Romania). The genomic DNA extract was stored at −4°C and used as template for all PCR experiments (Preda et al., 2021).

The genotypic virulence profile for 24 MRSA strains was characterized by analyzing the presence of genes encoding eight virulence factors, intensively expressed in these selected strains.

The protocol developed by Cotar et al. (2010) was followed, respecting the sequences of the primers used as well as the reaction parameters. Several virulence genes were detected by Polymerase Chain Reaction (PCR) through uniplex/simplex tests (*fnbA* gene coding, fibronectin adhesin A; *coag* gene, coagulase enzyme) and multiplex tests (*clf*A and *clf*B genes, bacterial surface adhesins; *fnb*B gene, fibronectin binding protein B; *fib* gene, fibrinogen binding protein; *bbp* gene, bone sialoprotein–binding protein; *ebpS*, elastin binding protein; Cotar et al., 2010). The reaction mix used was GoTaq® Green Master Mix (Jena Bioscience, Germany; Cotar et al., 2010).

2.3 Statistical analysis

The obtained results were analyzed statistically. The correlation between the continuous variables was tested by estimating the Pearson linear correlation coefficients (r) according to the established criteria. Correlation coefficients can take values between −1 and 1, showing a negative correlation (between −1 and 0) or a positive correlation

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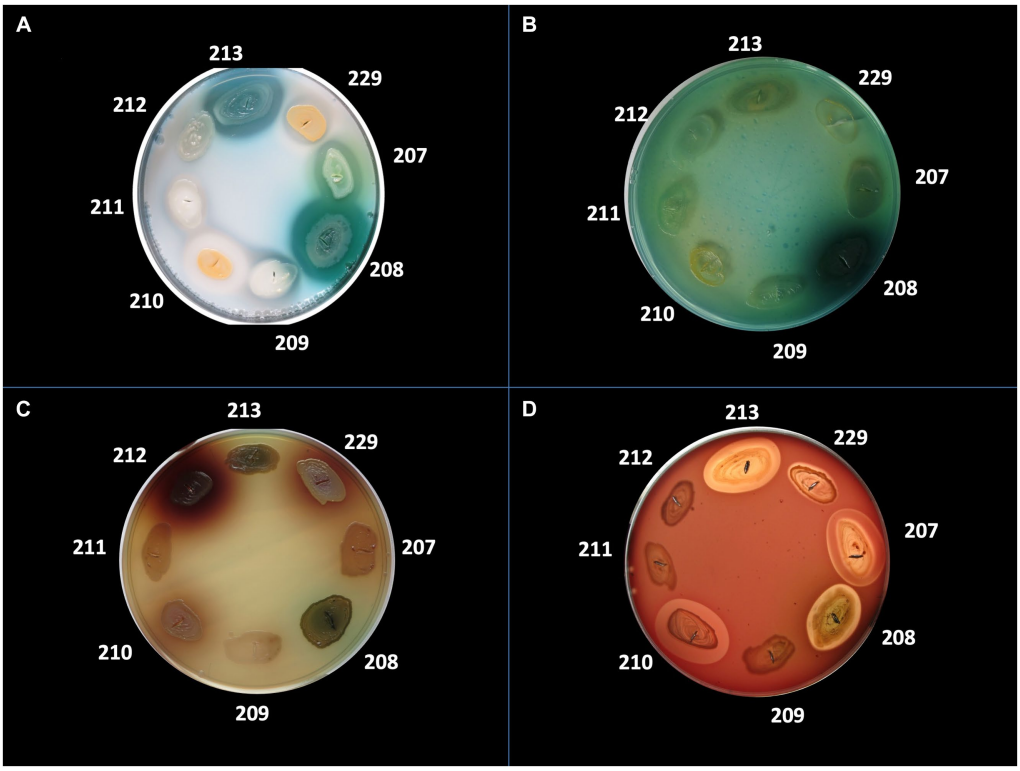

FIGURE 2
Detection of the production of different virulence factors in 8 bacterial strains analyzed. **(A)** Virulence factor: caseinase, medium: agar with addition of 15% casein; **(B)** Virulence factor: DNase, medium: DNase; **(C)** Virulence factor: esculinase, medium: agar supplemented with 1% esculin and iron citrate; **(D)** Virulence factor: hemolysins, medium: agar supplemented with 5% sheep blood. Bacterial strains tested: no. 207 *Pseudomonas aeruginosa*, no. 208 *P. aeruginosa*, no. 209 *Escherichia coli*, no. 210 *Staphylococcus aureus*, no. 211 MRSA, no. 212 *Klebsiella pneumoniae*, no. 213 *P. aeruginosa*.

(between 0 and 1). Statistical analysis was performed using Excel and Analyse-it (Method Validation Edition) software.

3 Results

3.1 Characteristics of the host

3.1.1 General characteristics of the patients' cohort

The study cohort was represented by 80 patients diagnosed with chronic skin wounds split in group no. 1 of 39 venous ulcers and group no. 2 of 41 other types of chronic skin wounds (7 arterial ulcers, 6 arterio-venous ulcers, 4 pressure sores, 3 post-surgical wounds, 10 wounds associated with abscesses, 2 paraneoplastic ulcerations, 9 ulcerations in the context of autoimmune vesiculo-bullous diseases) hospitalized in the "Elias" Emergency University Hospital, Bucharest, Romania, during the last 5 years.

From the total number of patients, 42 were females, 38 males, with a mean age of 62.1 and median age of 65.25 years old, while in the group suffering from chronic venous ulcers there were 23 female and 16 male patients, with a mean age of 69.1 and median age of 70 years old (Table 1).

It was registered a total number of 147 hospitalizations of the enrolled patients, more frequent during summer compared to other seasons (67 vs. 80), with an average of two times per patient and with a maximum of 14 hospitalizations for chronic venous wounds.

Regarding the comorbidities, in the group suffering from chronic venous ulcers the most frequent ones were essential hypertension (72%), ischemic cardiomyopathy (56.4%), dyslipidemia (41%), and type I/II diabetes (38.5%). 46.2% had a history of deep vein thrombosis of the lower limbs. The prevalence of comorbidities in patients diagnosed with venous ulcers and with other types of chronic lower limb wounds (arterio-venous ulcer, arterial ulcer) were comparatively analyzed. Patients with arterio-venous ulcers presented in a significantly higher proportion cardiac arrhythmia (66.7% vs. 30.8%), cardiac valvopathy (66.7% vs. 35.9%), ischemic cardiomyopathy (66.7% vs. 56.4%), hepatic steatosis (16.7% vs. 7.7%) and less frequently diabetes mellitus (16.7% vs. 38.5%), dyslipidemia (33.3% vs. 41%). Compared to patients diagnosed with chronic venous ulcers, patients with arterial ulcers had a significantly higher frequency of hepatic steatosis (28.6% vs. 7.7%), type I/II diabetes (42.9% vs. 38.5%), dyslipidemia (42.9% vs. 41%) and less hypertension (28.6% vs. 72%), ischemic cardiomyopathy (42.9% vs. 56.4%), cardiac arrhythmia (14.3% vs. 30.8%), and cardiac valvulopathy (28.6% vs. 35.9%).

3.1.2 Clinical characteristics of the wounds

3.1.2.1 Wound size

Depending on their size, the wounds were classified according to the RESVECH questionnaire, in the following six intervals:

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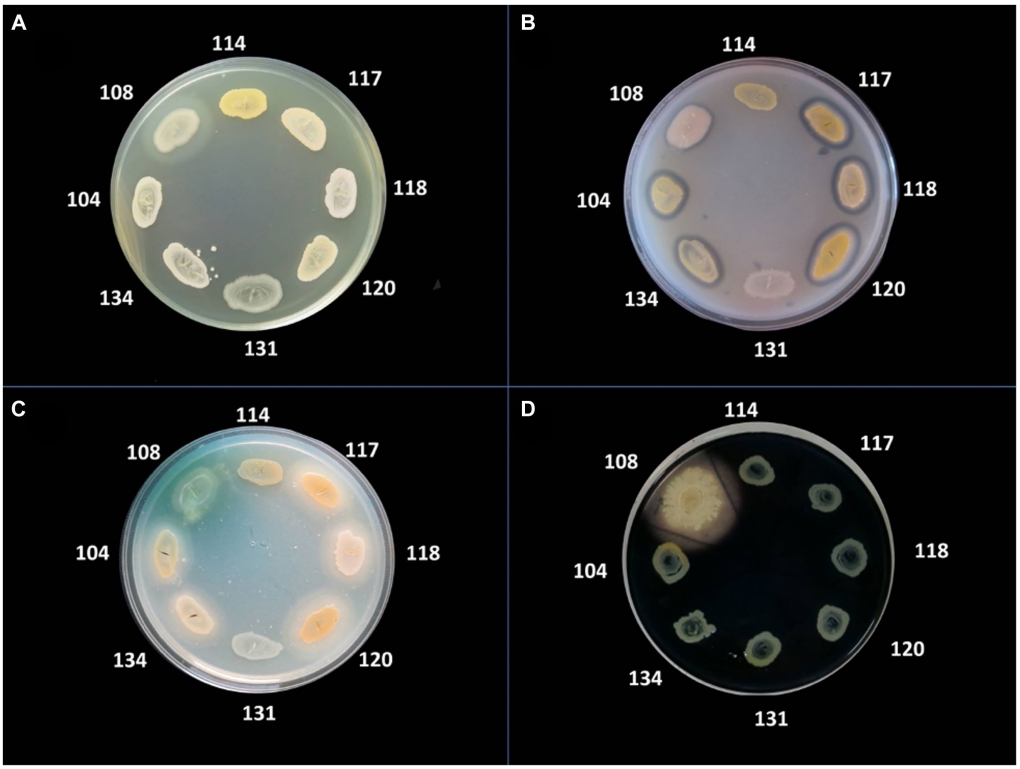

FIGURE 3
Detection of the production of different virulence factors in 8 bacterial strains analyzed. **(A)** Virulence factor: gelatinase; medium: agar with the addition of 3% gelatin; **(B)** Virulence factor: lecithinase, medium: agar with the addition of 2.5% egg yolk; **(C)** Virulence factor: lipase, medium: agar supplemented with 1% Tween 80; **(D)** Virulence factor: amylase, medium: agar with 1% starch added, flooded with Lugol solution. Bacterial strains tested: no. 104 *Staphylococcus aureus*, no. 108 *Pseudomonas aeruginosa*, no. 114 MRSA, no. 117 *S. aureus*, no. 118 *S. aureus*, no. 120 *S. aureus*, no. 131 *S. aureus*, no. 134 MRSA.

TABLE 1 Mean, median, and standard deviation values of age in patients depending on diagnosis and gender.

All types of ulcers	Total (n = 80)			Female (n = 42)			Male (n = 38)		
	Mean	Median	SD	Mean	Median	SD	Mean	Median	SD
Age	62.1	65.25	18.6	61.23	64	19.16	62.97	66.5	18.17

Venous ulcers	Total (n = 39)			Female (n = 23)			Male (n = 16)		
	Mean	Median	SD	Mean	Median	SD	Mean	Median	SD
Age	69.1	70	12.55	69.04	70	12.36	69.18	68	13

Other types of chronic skin wounds	Total (n = 41)			Female (n = 19)			Male (n = 22)		
	Mean	Median	SD	Mean	Median	SD	Mean	Median	SD
Age	55.36	59	21.02	51.78	49	21.97	58.45	63.5	20.16

1 = 0.1-4 cm^2, 2 = 4–15.9 cm^2, 3 = 16–35.9 cm^2, 4 = 36–63.9 cm^2, 5 = 64–99.9 cm^2, 6= > 100 cm^2. 15 patients presented venous ulcerations larger than 16 cm^2 and only one venous ulcer exceeded 100 cm^2. The majority of the patients with chronic venous ulcers were included in the first three intervals, with the highest number in the second one of 4–15.9 cm^2 (Table 2).

3.1.2.2 RESVECH score

The RESVECH score (minimum value 0, maximum value 40) in patients with chronic venous ulcers (Group 1) ranged from 12 to 34, with a mean of 21.61 and a median of 19. The venous wounds were exceeded in severity by arterial ulcers with a median RESVECH of 21.5, by arterio-venous ulcers with 24, and pressure sores with 25,

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TABLE 2 Wound size classified in six intervals.

Wound size	0.1–4 cm²	4–15.9 cm²	16–35.9 cm²	36–63.9 cm²	64–99.9 cm²	>100 cm²	Total
Venous ulcer	7	17	9	2	3	1	39
Arterial ulcer	0	5	1	0	5	0	11
Arterio-venous ulceration	1	2	2	0	0	1	6

values correlated with the median surface area of each type of lesion and with the median evolution (Table 3). The Pearson linear correlation test of the size of the ulceration and the RESVECH score, in patients with venous ulcers, issued a correlation coefficient $r = 0.837 > 0.28$, highlighting an intensely positive correlation between the two variables.

3.1.3 Systemic inflammatory response

Although the inflammatory biological syndrome was present in 59% of the patients diagnosed with chronic venous ulcers (Group 1), only 20.5% associated leukocytosis with neutrophilia. Compared to venous ulcers, in the case of other types of chronic skin lesions (Group 2) a lower proportion of biological inflammatory syndrome (51.2%) was observed, to which it was associated more frequently (approximately twice) leukocytosis with neutrophilia (41.5%). This may reflect the fact that the host response to infection may be more severe in other types of chronic skin lesions compared to venous ulcers. The Pearson test of the linear correlation demonstrated a weak statistically positive correlation between the presence of the biological inflammatory syndrome detected at the initial monitoring and the evolution of skin wounds, with a Pearson r value of $0.234 < 0.28$.

3.1.4 Evolution of disease

One third of the total number of patients diagnosed with chronic venous ulcers (Group 1; 33.33%) was cured (Figure 4), this proportion being significantly higher compared to that observed in leg ulcers of other etiology, as well as in the case of arterial wounds (14.28%), mixed etiology (0%). However, 71.4% of patients with arterial ulcers had a favorable evolution, with a reduction in the RESVECH score. Ulcers of mixed etiology (arterio-venous) presented a more severe evolution, with stationary or aggravated lesions at the end of the monitoring period.

A higher proportion of severe septicemic events resulting in death were observed in patients diagnosed with other types of chronic skin lesions (Group 2) compared to patients with venous ulcers (Group 1; Weller et al., 2021; Stanek et al., 2023).

In patients with venous leg ulcers (Group 1), the Pearson test of linear correlation did not demonstrate a statistically relevant correlation between the evolution of the ulceration at the end of monitoring and its initial size (Pearson $r = 0.052$), respectively the initial RESVECH score (Pearson $r = 0.037$).

3.2 Microbiological characterization

3.2.1 Strain identification

A total number of 104 bacterial strains were isolated from 80 patients diagnosed with chronic skin lesions, admitted to the "Elias" Emergency University Hospital, Bucharest, Romania, during the last 5 years. 49 strains were isolated from patients with chronic venous ulcers (Group no. 1).

TABLE 3 Mean, median, and standard deviation values of the RESVECH score in patients depending on diagnosis.

	RESVECH score		
	Mean	Median	SD
Venous ulcers ($n = 39$)	21.61	19	4.88
Arterial ulcers ($n = 7$)	22.14	21.5	3.38
Arterio-venous ulcers ($n = 6$)	24.6	24.5	6.28
Pressure sore ($n = 4$)	25	25	0

During the entire period all 104 isolated strains were identified as belonging to seven bacterial species as follows: the most common was *S. aureus* (78 strains, 75%), followed by *P. aeruginosa* (12 strains, 11%) and the enterobacteria *K. pneumoniae* (5 strains, 5%), *E. coli* (4 strains, 4%), *M. morganii* (2 strains, 2%) *P. mirabilis* (1 strain, 1%), *C. freundii* (1strain, 1%) and β-hemolytic streptococci of group G (1 strain, 1%; Figure 5; Table 4).

3.2.2 Phenotypic assessment of the virulence profile of microorganisms

3.2.2.1 Phenotypic assessment of the adherence to human cell substrate

Adherence to living or inert substrate and the formation of bacterial aggregates are essential steps in the biofilms' development (Donlan and Costerton, 2002). Testing the ability of bacterial strains isolated from delayed-healing skin lesions to adhere to human cell substrate was the first step in defining the potential of microorganisms to trigger chronic infections, with an impact on the physiological process of skin healing. For 44 bacterial strains the cell substrate used was HeLa cell line (Figure 1; Table 5), while for the other 60 strains the cellular substrate used was represented by endothelial diploid cell cultures (Table 6).

S. aureus strains presented an increased adherence capacity to human cell substrate human cell substrate, both for HeLa and endothelial cells, with an average of the adherence index of over 50% for methicillin-sensitive *S. aureus* (MSSA) and over 70% for MRSA, respectively, over 22.42% for MSSA and over 31.14% for MRSA (Tables 2, 3). So, MRSA strains showed the highest adherence to cellular substrate compared to MSSA strains, but also other bacterial strains belonging to other species showed this property.

It was observed that MRSA and *P. aeruginosa* strains showed the highest degree of adherence to the human cell substrate and their

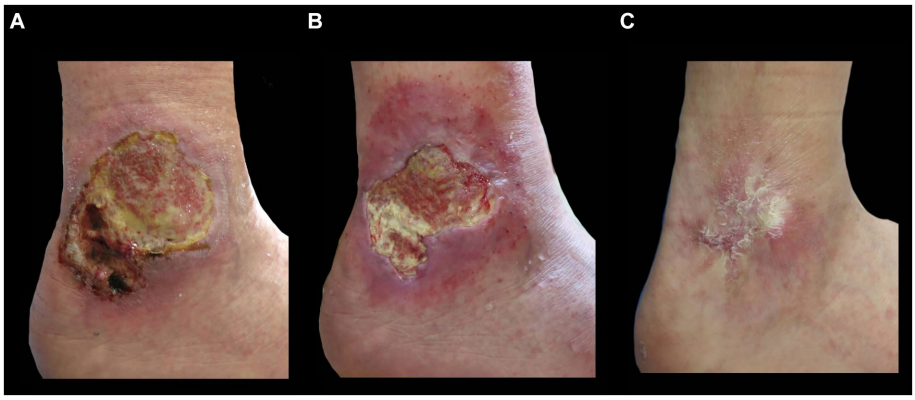

FIGURE 4
Favorable evolution of a patient diagnosed with chronic venous leg ulcer. **(A)** Initial clinical aspect of the wound. **(B)** Clinical aspect at 2 months. **(C)** Clinical aspect at 6 months.

possible association in polymicrobial infections could influence the persistence of the infection at the level of venous ulcers.

Compared to *S. aureus* and *P. aeruginosa* strains, the adherence of enterobacteria to human cell substrate was significantly lower quantitatively, the adherence index to HeLa cells being between 0 and 50%, respectively between 0 and 30% to endothelial cells. Their adherence pattern was also suggestive of the strains' reduced ability to develop bacterial aggregates. If *E. coli* and *P. mirabilis*, isolated from lower limb venous and arterial ulcers adhered locally or diffusely, in minimal proportions to the substrate 0%–20%, the strains of *C. freundii* and *K. pneumoniae*, isolated from venous ulcers, showed a diffuse or diffuse aggregative adherence to HeLa cells of 30–50% (Table 2). This result does not exclude the involvement of *C. freundii* and *K. pneumoniae* in the development of chronic mono- or polymicrobial infections in venous ulcers.

An interesting observation concerns the fact that the strains tested showed a preferential type of adherence depending on the isolation source. The diffuse aggregative subtype of adherence to human cells was observed more frequently in strains isolated from chronic lower limb ulcers (venous or of other etiology), compared to other types of chronic skin lesions (Supplementary Figures 1, 2). This adherence pattern suggests the great potential of microorganisms to develop cellular aggregates and implicitly biofilms.

The adherence index was high for all sources, with the highest values recorded to HeLa cells of 92.5% for lower limb ulcers that exclude venous etiology (Supplementary Figure 3), and to endothelial cells of 60.7%, for venous ulcers (Supplementary Figure 4).

The strains isolated from venous ulcers showed a high degree of adherence to HeLa cell substrate, regardless of the observed adherence pattern. These results are also reflected by the value of the average HeLa index established only according to the isolation source, which was almost two times higher in strains isolated from venous ulcers (46%) compared to those isolated from leg ulcers of other etiology (25%) or other types of chronic skin lesions (25%). The average index of adherence to endothelial cells was also higher in strains isolated from venous ulcers (24%) compared to those isolated from leg ulcers of other etiology (16%) or other types of chronic skin lesions (21%).

3.2.2.2 Phenotypic assessment of biofilm development *in vitro*

All strains of *S. aureus* developed biofilms on the inert substratum, with variations observed depending on the source of isolation. Strains isolated from arterial and artero-venous ulcers showed the lowest ability to form such protective structures. The strains isolated from venous ulcers developed biofilms with a medium intensity, while staphylococci from other types of chronic skin lesions produced biofilms with the highest intensity. The spectrophotometric quantification of the biomass of *S. aureus* did not demonstrate significant differences between MSSA and MRSA isolates from lower limb ulcers, regardless of etiology. On the other side, MRSA strains isolated from other types of chronic skin lesions showed a more intense ability to form biofilms, compared to MSSA strains. Quantitative detection revealed maximum values after culture plate incubation periods of 24 and 48 h. After a 72 h of incubation, the large mass of biofilms becomes more fragile, thus detaching more easily from the surface of the micro-wells during the fixation and staining processes.

All strains of *P. aeruginosa* developed biofilms, with a significant difference between the strains isolated from venous ulcers compared to the strains that came from other sources (they produced more intensively such structures). Strain no. 7 produced the most intense biofilm. Compared to *S. aureus* strains the production of biofilms was similar. Strain no. 16, characterized by an increased ability to develop biofilms, was isolated from a patient diagnosed with chronic venous ulcer, who died of sepsis; this strain showed a complex virulence profile by the production of pore-forming toxins, DN-ase, and by an intense diffuse aggregative adherence to HeLa cells and an adherence index of 100%, as well as multiple antibiotic resistance.

The ability to develop biofilms of *Enterobacteriaceae* strains was significantly lower compared to *S. aureus* and *P. aeruginosa* strains. This result is consistent with the analysis of adherence to HeLa cell or endothelial cell substrate, inferior in *Enterobacteriaceae* compared to *S. aureus* and *P. aeruginosa* strains. Compared to other enterobacteria, *K. pneumoniae* strains showed both greater adhesion to cellular substrate, as well as more intense biofilm development.

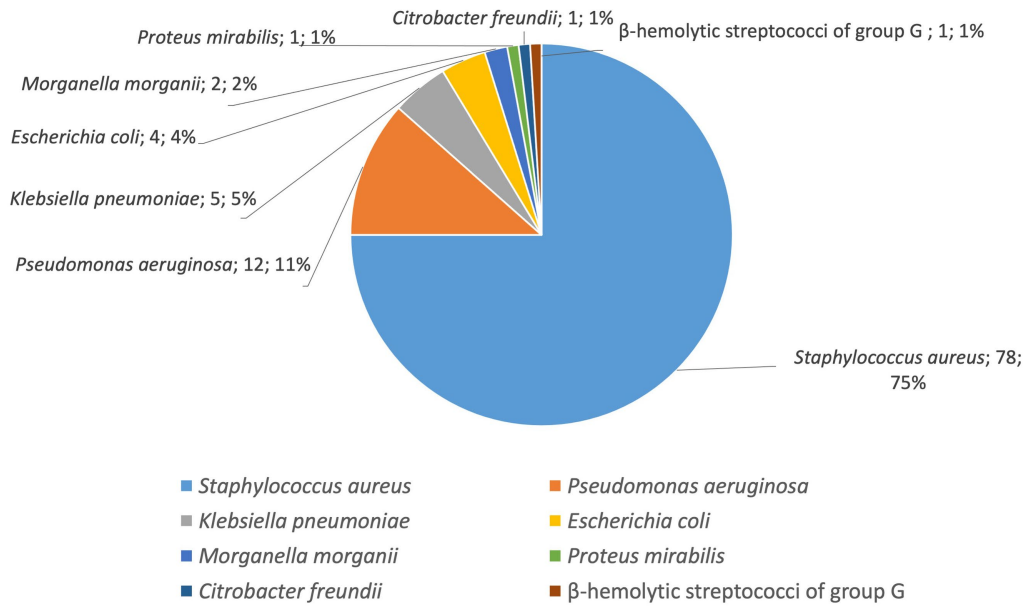

FIGURE 5
Distribution by species of the isolated bacterial strains (number of strains, %).

Besides testing the ability of each strain to develop biofilms, this virulence characteristic was also evaluated for the polymicrobial association of strains isolated from the same sample. Thus, the development of biofilms was studied for the following microorganisms: 4 bimicrobial associations, 3 of which were represented by *P. aeruginosa* and MRSA and a bimicrobial association of *P. aeruginosa* and MSSA, as well as an association of 3 bacterial species: *P. aeruginosa*, MSSA, and *M. morganii*.

Where MRSA and *P. aeruginosa* strains isolated from arterio-venous lower limb ulcers were inoculated together, the formation of biofilms was observed with a much-increased intensity, up to 5–6 times higher. In polymicrobial infections, *P. aeruginosa* has the ability to increase the virulence of biofilms by enhancing the development of other bacterial species (Scales and Huffnagle, 2013). Regarding the associations of strains originating from venous ulcers, in both bimicrobial and trimicrobial biofilms, no significant increases were observed compared to individual strains.

3.2.2.3 Phenotypic assessment of the production of soluble virulence factors

The phenotypic virulence profiles of different bacterial species were analyzed and compared, by cultivating isolated strains on special media containing the enzyme substrate corresponding to each soluble virulence factor: pore-forming toxins (lecithinase, lipase, hemolysins) and exotoxins (caseinase, gelatinase, amylase, DN-ase), esculinase (Figures 2, 3).

Regarding the distribution of virulence factors in *S. aureus* strains, it was observed that lecithinase was the most frequently expressed virulence factor (62 strains, 80.5%), followed, in approximately equal proportions, by lipase (55 strains, 71.4%), hemolysins (56 strains, 72.7%), and esculinase (56 strains, 72.7%). To a lesser extent, DN-ase (34 strains, 44.2%), caseinase (35 strains, 45.5%), and amylase (32 strains, 41.6%). Gelatinase was the least expressed virulence factor in the tested strains. Thus, *S. aureus* strains predominantly expressed pore-forming toxins (lecithinase, lipase, and hemolysins), necessary for bacterial multiplication and dissemination. Hemolysins are also involved in increasing the supply of iron, necessary for the activation of microbial genes and the expression of other virulence factors (Table 7).

Comparing the phenotypic virulence profile of MSSA vs. MRSA strains, significant differences in the production of virulence factors were observed. MSSA strains showed more often complete hemolysis (1.7/1), DNase (2.2/1), amylase (1.7/1), lecithinase (1.2/1) and less often incomplete hemolysis (1/1.5), gelatinase (1/2.6), esculinase (1/1.2), caseinase (1/1.3). Lipase was expressed in approximately equal proportions. MRSA strains, compared to MSSA strains, expressed, along with pore-forming toxins, more intensively the enzymes involved in the degradation of the extracellular matrix, devitalized tissue and debris, such as caseinase and gelatinase (which belongs to matrix metaloproteinases).

The virulence profile for each bacterial species was compared, depending on the source of isolation: venous ulcer, lower limb ulcer of other etiology and other types of chronic skin lesions.

From the point of view of the production of hemolysins, the strains that came from lower limb ulcers of vascular etiology (venous, arterial, mixed), regardless of the bacterial species, showed a more frequent expression of β-hemolysins. Moreover, *Enterobacteriaceae* strains from venous ulcers expressed alpha-hemolysins significantly more frequently compared to the isolated from other sources (75% vs. 50 and 33.3%).

The strains that came from lower limb ulcers of vascular etiology (venous, arterial, mixed), compared to those from other chronic skin lesions, also showed the following differences in terms of the frequency of production of virulence factors: increased production of gelatinase in *Enterobacteriaceae* but lower in *P. aeruginosa*, decreased esculinase production in *Enterobacteriaceae* but increased in *P. aeruginosa*, decreased lecithinase production in *P. aeruginosa*. For strains isolated from venous ulcers, compared to microorganisms that came from other sources, a more frequent expression of lipase and amylase was observed in MRSA and *P. aeruginosa*. For strains of MSSA and

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TABLE 4 Strains isolated in the study and their sources.

	MSSA	MRSA	*P. aeruginosa*	*K. pneumoniae*	*E. coli*	*M. morganii*	*P. mirabilis*	*C. freundii*	Group G β-hemolytic streptococci
Venous ulcer	21 strains (5, 8, 18, 21, 28, 31, 34, 44, 104, 120, 131, 145, 146, 166, 169, 203, 205, 210, 215, 216, 226)	12 strains (11, 20, 27, 36, 43, 140, 157, 163, 206, 220, 221, 228)	8 strains (7, 16, 37, 39, 108, 164, 207, 213)	2 strains (4, 177)	3 strains (13, 42, 209)	2 strains (214, 218)	0	1 strain (1)	1 strain (9)
Arterial ulcer	5 strains (29, 118, 165, 219, 223)	1 strain (22)	0	0	1 strain (23)	0	1 strain (17)	0	0
Arterio-venous ulcer	0	8 strains (30, 156, 170, 176, 183, 201, 217, 227)	3 strains (143, 181, 202)	0	0	0	0	0	0
Pressure sore	1 strain (35)	4 strains (19, 25, 126, 136)	0	0	0	0	0	0	0
Post-surgical wound	1 strain (14)	1 strain (2)	0	1 strain (3)	0	0	0	0	0
Wounds associated with abscesses	9 strains (6, 10, 26, 32, 33, 38, 40, 41, 117)	5 strains (12, 107, 134, 147, 167)	0	0	0	0	0	0	0
Paraneoplastic ulcerations	2 strains (15, 24)	0	0	0	0	0	0	0	0
Ulcerations in autoimmune vesiculo-bullous diseases	1 strain (204)	7 strains (114, 125, 168, 211, 224, 225, 229)	1 strain (208)	2 strains (180, 212)	0	0	0	0	0

Enterobacteriaceae this proportion was reversed. DNase was produced only by *P. aeruginosa* strains originating from venous ulcers.

Pseudomonas aeruginosa strains isolated from other types of chronic skin lesions intensively produced gelatinase and caseinase, enzymes involved in local invasiveness, since they degrade the extracellular matrix of the connective tissue. The spectrum of soluble virulence factors of *P. aeruginosa* strains isolated from venous ulcers was different from that of strains isolated from other sources, suggesting the existence of some damaging factors that could modify the virulence profile of microorganisms. Moreover, *Enterobacteriaceae* from venous ulcers expressed virulence factors involved in local invasiveness (caseinase, gelatinase), but also in the dissemination of infection (alpha-hemolysins).

According to the average virulence index calculated, a similar intensity of expression of virulence factors was observed in *S. aureus* strains isolated from non-venous lower limb ulcers and from other types of chronic skin lesions. The virulence of MRSA strains isolated from venous ulcers was slightly higher compared to those from other sources (ratio of 1.12/1/1). *Pseudomonas aeruginosa* strains and those belonging to the *Enterobacteriaceae* family isolated from other types of chronic skin lesions had a more intense virulence profile compared to those isolated from venous or leg ulcers.

3.2.3 Genotypic assessment of virulence genes of MRSA strains by polymerase chain reaction

The study characterized the phenotypic and genotypic virulence profile of 24 MRSA selected strains isolated from chronic skin lesions (12 strains from chronic venous ulcers) of patients admitted to the Dermatology Clinic, "Elias" University Emergency Hospital, Bucharest (Table 8).

The studied strains showed a variable ability to adhere to the cell substrate and develop biofilms, observing a statistically significant linear correlation between the two studied properties. The analysis of the distribution of extracellular virulence factors demonstrated that the pore-forming toxins, hemolysins, lipase, and lecithinase, were produced, each in 75% of the MRSA strains analyzed, in approximately equal proportions depending on the location source. This highlights the increased potential for systemic dissemination of MRSA strains. Caseinase and gelatinase exoproteases were produced by a smaller number of MRSA strains (45.8% and 25%, respectively). DNase was detected in 32% of the strains.

Phenotypic changes are most often correlated with a specific genotypic profile. So, the next experimental step was represented by genotypic screening aimed at identifying the presence or absence of certain genes encoding extracellular virulence factors or involved in invasiveness and adhesion.

The analysis of the distribution of virulence genes demonstrated that 23 of the 24 analyzed strains (95.8%) expressed genes encoding adhesins associated with the bacterial surface (*clfA* and *clfB*), 23 strains expressed the *coag* gene (95.8%), and 17 strains had expressed *fnbA* gene (70.8%). The *fnbA* gene was less frequently detected in strains isolated from venous ulcers, in a proportion of 50%, compared to 91.6% in the case of other sources of isolation (91.6%; arterial ulcers, arterio-venous ulcers, chronic surgical wounds, chronic abscesses). None of the MRSA strains analyzed expressed the genes *fnbB* (fibronectin-binding protein), *fib, bbp* and *ebpS* (elastin-binding protein; Table 8).

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TABLE 5 Adherence pattern to HeLa cell substrate for each isolated bacterial strain.

Adherence pattern to HeLa cells	No adherence	Localized	Diffuse	Localized aggregative	Diffuse aggregative	Adherence index
MSSA	2 strains (35, 38)	4 strains (6, 26, 29, 41)	4 strains (5, 15, 31, 44)	7 strains (8, 10, 18, 24, 28, 33, 40)	4 strains (14, 21, 32, 34)	0–100%
						Average: 52.38%
MRSA	0	0	1 strain (11)	5 strains (2, 12, 19, 20, 36)	5 strains (22, 25, 27, 30, 43)	10–100%
						Average: 73.18%
P. aeruginosa	0	0	2 strains (7, 39)	0	2 strains (16, 37)	10% (7), 80% (39), 100% (16, 37)
						Average: 63.33%
C. freundii	0	0	1 strain (1)	0	0	50% (1)
E. coli	1 strain (42)	1 strain (13)	1 strain (23)	0	0	0% (42), 10% (23), 20% (13)
						Average: 10%
K. pneumoniae	0	0	0	0	2 strains (3,4)	30% (3), 40% (4)
						Average: 35%
P. mirabilis	0	1 strain (17)	0	0	0	5% (17)

MSSA, methicillin-sensitive *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *E. coli*, *Escherichia coli*; *K. pneumoniae*, *Klebsiella pneumoniae*; *M. morganii*, *Morganella morganii*.

TABLE 6 Adherence pattern to endothelial cell substrate for each isolated bacterial strain.

Adherence pattern to endothelial cells	No adherence	Localized	Diffuse	Localized aggregative	Diffuse aggregative	Adherence index
MSSA	5 strains (131, 145, 146, 204, 226)	3 strains (118, 166, 169)	3 strains (117, 120, 205)	3 strains (104, 219, 223)	5 strains (165, 202, 203, 210, 215, 216)	0–85%
						Average: 22.42%
MRSA	2 strains (125, 225)	6 strains (107, 170, 183, 211, 221, 229)	8 strains (134, 136, 167, 176, 206, 220, 227, 228)	5 strains (114, 126, 147, 201, 217)	6 strains (140, 156, 157, 163, 168, 224)	0–90%
						Average: 31.14%
P. aeruginosa	1 strain (207)	0	1 strain (208)	3 strains (108, 164, 213)	3 strains (143, 181, 202)	0–40%
						Average:12.5%
E. coli	0	1 strain (209)	0	0	0	5% (209)
K. pneumoniae	0	1 strain (189)	0	2 strains (177, 212)	0	Average: 12.66%
						3% (177), 5% (180), 30% (212)
M. morganii	0	1 strain (218)	1 strain (214)	0	0	Average: (7.5%)
						5% (218), 10% (214)

MSSA, methicillin-sensitive *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *E.coli*, *Escherichia coli*; *K. pneumoniae*, *Klebsiella pneumoniae*; *M. morganii*, *Morganella morganii*.

3.3 Statistical analysis

No statistically significant correlation was found between the cell substrate adherence index of the strains isolated from venous ulcers and the initial RESVECH severity score of the wounds (Pearson's $r = -0.033$). Also, no positive or negative correlation was detected between the detection of a polymicrobial infection during the microbiological diagnostic examination and the ability of the isolated microorganisms to adhere to the cell substrate (Pearson's $r = 0.064$).

The test of the linear correlation between the index of adherence to the cell substrate and the average intensity of development of bacterial biofilms of the strains isolated from venous ulcers revealed a Pearson r coefficient of 0.456 (>0.2) and a statistically significant correlation. Thus, it was demonstrated that the adherence of bacterial strains to human cellular substrate correlates proportionally with their property to develop biofilms.

The following values were associated with each adherence pattern: localized adherence = 1, diffuse adherence = 2, aggregative localized adherence = 3, aggregative diffuse adherence = 4, depending on their potential to be associated with the development of biofilms. The test of the linear correlation between the pattern of adherence to the cell substrate and the average intensity of development of bacterial biofilms of the strains isolated from venous ulcers revealed a statistically significant correlation, with a Pearson r coefficient of 0.339. It was thus demonstrated that the pattern of adherence to the cellular substrate, including the property of microorganisms to form

TABLE 7 The distribution of virulence factors in *Staphylococcus aureus* strains.

Soluble virulence factor	*Staphylococcus aureus* (all strains)		MSSA		MRSA	
	No.	%	No.	%	No.	%
Alpha-hemolysin	22	28.6	9	22.5	13	35.1
Beta-hemolysin	34	44.2	22	55.0	12	32.4
Lipase	55	71.4	29	72.5	26	70.3
Lecithinase	62	80.5	35	87.5	27	73.0
Esculinase	56	72.7	26	65.0	30	81.1
DN-ase	34	44.2	24	60.0	10	27.0
Caseinase	35	45.5	16	40.0	19	51.4
Gelatinase	17	22.1	5	12.5	12	32.4
Amylase	32	41.6	21	52.5	11	29.7
Total no. of virulence factors	347		187		160	

MSSA, methicillin-sensitive *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*.

cellular aggregates, correlated proportionally with the property of MRSA to develop biofilms.

Statistical tests revealed a weak positive correlation between the average intensity of bacterial biofilm development of strains isolated from venous ulcers and the initial RESVECH severity score of the lesions, with a Pearson r coefficient of $0.203 < 0.28$. Considering the multitude of factors that influence the severity of venous ulcers such as host comorbidities, vascular deficiency, the correctness of treatment administration, the type of treatment used and many others, this result does not exclude the relevance of the pathogenic hypothesis. Further studies are needed to establish whether there is a correlation between the intensity of bacterial biofilm development and the severity of the venous ulcer lesion.

There was no correlation between the mean intensity of development of bacterial biofilms in strains isolated from venous ulcers and the final evolution of the patients (healing = 1, improvement = 2, stagnation = 3, aggravation = 4, death = 5; Pearson's $r = -0.011$). The evolution of patients' injuries can be influenced by multiple factors, including the correctness of administering the recommended treatment at home.

4 Discussion

In an unique attempt in international scientific research, within the experimental study, we characterized phenotypically and molecularly the virulence profiles of bacterial strains isolated from chronic venous ulcers, as well as from other types of chronic wounds associated with difficult to treat infections. We aimed to discover clinical-microbiological correlations, obtaining results that might contribute to the elucidation of the impact of the microbiota on delayed wound healing.

Studies of the cutaneous microbiome conducted *in vitro* and *in vivo* have confirmed the widespread belief that the microorganisms colonizing skin wounds affect their healing. Contradictory results have emerged from these studies, nevertheless. In their *in vivo* study on germ-free Swiss mice, Canesso et al. (2014) showed rapid and scarless wound healing in the absence of commensal skin microbiota. Additionally, neutrophil accumulation was decreased, anti-inflammatory cytokine levels were elevated, and vascular endothelial growth factor (VEGF) was upregulated (Canesso et al., 2014). Their wound healing characteristics resembled those of standard mice after the skin microbiota had been restored, indicating that the commensal bacteria might have a negative impact (Canesso et al., 2014), because they, especially staphylococci, can behave as opportunistic pathogens. On the other hand, it was discovered that animals receiving oral Vancomycin had a lower bacterial density of skin wounds and slower wound healing rates due to the impact on keratinocyte differentiation and proliferation by the downregulation of the proteins interleukin (IL)-17 and regenerating islet derived protein-III gamma (RegIIIy; Zhang et al., 2015; Johnson et al., 2018).

Bacteria are able of intercellular communication and further, to sense and rapidly adapt to their environment through coordinated, multi-cellular responses. Even in human wounds, they are able to sense, respond, and manipulate host-immune responses, toward local persistence of infection and, in particular circumstances, toward systemic invasion and septicemic events (Chifiriuc et al., 2022).

The evolution of patients with chronic, superinfected venous ulcers was less severe compared to that of patients with decubitus ulcers or chronic leg ulcers of other etiology (arterial or mixed). This result could be determined by the existence of multiple decompensated comorbidities in the latter, but also by different bacterial virulence phenotypes.

Adherence to living or inert substrate and the production of bacterial aggregates are essential steps in the development of biofilms (Donlan and Costerton, 2002). Testing the ability of bacterial strains isolated from delayed-healing wounds to adhere to human cellular substrate was the first step in defining the potential of microorganisms to trigger chronic infections, with an impact on the physiology of skin healing.

Bacterial strains isolated from chronic venous ulcers, but also from other types of chronic lower limb ulcers, showed high intensity adherence to human cellular substrate, of diffuse aggregative type, statistically associated with the microorganisms' ability to develop bacterial biofilms (Pearson $r\ 0.456 > 0.28$).

MRSA strains showed a more significant ability to adhere and aggregate to human cell substrate (both HeLa cells and endothelial cells) compared to other bacterial species studied and, implicitly, an increased potential to develop bacterial biofilms. Along with the multi-resistance of MRSA to antibiotics, the increased tolerance of biofilms to the action of antimicrobial molecules may further reduce the variety of effective therapeutic options. It was observed that MRSA and *P. aeruginosa* strains showed the highest degree of adherence to the cellular substrate and their possible association in polymicrobial infections could influence the persistence of infection in venous ulcers, as well as other types of wounds.

Compared to *S. aureus* and *P. aeruginosa* strains, the adherence of the studied enterobacteria to the HeLa or endothelial cell substrates was significantly less quantitatively. A possible explanation may be that the planktonic, free phenotype is probably more characteristic for these microorganisms. The presence of biological inflammatory syndrome in patients with *Enterobacteriaceae* wound isolates could be explained precisely by the potential of the planktonic phenotype to

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TABLE 8 Results of the genotypic assessment of 8 virulence genes in MRSA strains isolated from chronic wounds, through Polymerase Chain Reaction uniplex/simplex tests (*fnbA* gene coding, fibronectin adhesin A; *coag* gene, coagulase enzyme) and multiplex tests (*clf*A and *clf*B genes, bacterial surface adhesins; *fnb*B gene, fibronectin binding protein B; *fib* gene, fibrinogen binding protein; *bbp* gene, bone sialoprotein–binding protein; *ebpS*, elastin binding protein; Cotar et al., 2010).

No.	Strain code	Isolation source	Virulence encoding genes							
			clf A	*clfB*	*fnbB*	*fib*	*fnbA*	*coag*	*bbp*	*ebpS*
1	11	Venous ulcer	+	+	–	–	–	+	–	–
2	20	Venous ulcer	+	+	–	–	+	+	–	–
3	27	Venous ulcer	+	+	–	–	+	+	–	–
4	36	Venous ulcer	+	+	–	–	+	+	–	–
5	43	Venous ulcer	+	+	–	–	+	+	–	–
6	140	Venous ulcer	+	+	–	–	–	+	–	–
7	157	Venous ulcer	+	+	–	–	–	–	–	–
8	163	Venous ulcer	–	–	–	–	+	+	–	–
9	206	Venous ulcer	+	+	–	–	–	+	–	–
10	220	Venous ulcer	+	+	–	–	+	+	–	–
11	221	Venous ulcer	+	+	–	–	–	+	–	–
12	228	Venous ulcer	+	+	–	–	–	+	–	–
13	114	Wound-autoimmune vesiculo-bullous diseases	+	+	–	–	+	+	–	–
14	136	Pressure sore	+	+	–	–	+	+	–	–
15	156	Arterio-venous ulcer	+	+	–	–	+	+	–	–
16	168	Wound-autoimmune vesiculo-bullous diseases	+	+	–	–	+	+	–	–
17	176	Arterio-venous ulcer	+	+	–	–	+	+	–	–
18	183	Arterio-venous ulcer	+	+	–	–	+	+	–	–
19	2	Surgical wound	+	+	–	–	+	+	–	–
20	12	Wound associated with abscess	+	+	–	–	+	+	–	–
21	19	Pressure sore	+	+	–	–	+	+	–	–
22	22	Arterial ulcer	+	+	–	–	+	+	–	–
23	25	Pressure sore	+	+	–	–	–	+	–	–
24	30	Arterio-venous ulcer	+	+	–	–	+	+	–	–

trigger a more intense inflammatory response. *Klebsiella pneumoniae* strains, being capsulated, were the most adherent enterobacteria.

An interesting observation is the identification of a particular pattern of adherence depending on the isolation source. The strains isolated from chronic lower limb ulcers of vascular etiology, compared to chronic skin lesions of other etiology, were more frequently associated with the diffuse aggregative pattern of adherence, of high intensity, to both types of cells used as substrate. This adherence pattern suggests the maximum potential of microorganisms to develop cellular aggregates and implicitly biofilms. Moreover, the strains isolated from venous ulcers showed a high degree of adherence to the cellular substrate for each pattern observed, more intense compared to the strains isolated from leg ulcers of other etiology or other types of chronic skin lesions. These results may explain the persistence of the infection and the reduced response to antimicrobials.

Adapting therapeutic management by using molecules that disrupt the adherence of microorganisms to the cellular substrate within lower limb ulcers could contribute to a lower infectious burden and faster healing rates.

Bacterial adherence to the endothelial cell substrate *in vitro* has been shown to be involved both in the systemic dissemination of infections and in the etiopathogenic mechanisms of severe conditions such as acute endocarditis (Ogawa et al., 1985; Claes et al., 2018). Our study revealed that the adherence to the endothelial cell substrate of the strains isolated from chronic skin lesions, regardless of etiology, was much reduced compared to the adhesion to the HeLa cell substrate. This could explain the rarity of developing severe systemic infections in patients with chronic wounds. However, MRSA strains showed a more significant ability to adhere and aggregate to the endothelial cell substrate compared to other bacterial species studied and therefore we emphasize the increased risk of the patients included in the study to develop severe infectious complications, being elderly patients, with multiple cardiovascular comorbidities.

The next stage of the study was represented by testing the ability of microorganisms to produce biofilms, structures compared by some authors to some "multi-cellular, primitive organisms" (Hoiby et al., 2010). Bacterial biofilms are "structurally and dynamically complex biological systems" (Lurie et al., 2020), composed of mono- or polymicrobial communities embedded in a protective extracellular matrix, which gives bacteria tolerance to antibiotics and to host's immune defense mechanisms (Mihai et al., 2015). The main characteristic of chronic wounds is the unfavorable evolution, even if the treatment regimen is in accordance with international guidelines. Most studies support a correlation between the presence of bacterial biofilms and the chronicity of skin lesions, but there are authors who doubt this hypothesis (Thomson, 2011; Percival et al., 2012, 2014).

All isolated strains demonstrated the ability to develop biofilms, with a variable intensity depending on the bacterial species, but also on the source of isolation. For the strains isolated from venous ulcers, there was not identified a significant correlation between the average intensity of development of bacterial biofilms and the final evolution of the wounds, that may be influenced by multiple factors, including the compliance to treatment recommended at home.

All strains of *S. aureus* developed biofilms, with variations observed depending on the source of isolation. *Staphylococcus aureus* strains isolated from venous ulcers developed biofilms with a higher intensity compared to the strains from lower limb ulcers of arterial or mixed etiology. No significant differences were observed between MRSA and methicillin-susceptible strains.

Pseudomonas aeruginosa strains developed biofilms intensively but those isolated from venous ulcers produced these structures more intensively compared to the strains that came from other sources. The ability to develop biofilms of *Enterobacteriaceae* strains was significantly lower compared to *S. aureus* and *P. aeruginosa* strains.

Pluri-microbial inoculations of MRSA and *P. aeruginosa* strains with the same source of infection (leg ulcers of mixed etiology, chronically superinfected inverse psoriasis) were associated with a 5–6 times higher intensity of biofilm development compared to individual strains.

Pseudomonas aeruginosa showed less virulent phenotypes. Both the ability to develop biofilms of strains from the *Enterobacteriaceae* family and the degree of virulence were inferior to microorganisms from the species *S. aureus* and *P. aeruginosa*.

In the next step we analyzed and compared the phenotypic virulence profiles in different bacterial species, by cultivating the isolated strains on special media containing the enzyme substrate corresponding to each soluble virulence factor: pore-forming toxins (lecithinase, lipase, hemolysins) and exoenzymes (caseinase, gelatinase, amylase, DN-ase, esculinase). While the characteristics of invasiveness, tissue destruction, and infection dissemination are primarily associated with the soluble virulence factors secreted by bacteria, the development of biofilms is primarily associated with the persistence of infection, resistance, and tolerance to antimicrobials, as well as host immune defense mechanisms (Preda et al., 2021). The manifestation of these virulence traits may account for the severity of wound infections as well as for their chronicization, which makes them challenging to cure (Preda et al., 2021).

Pore-forming toxins such as lecithinase, lipase, hemolysins are involved in the mechanisms of spreading the infection. Lecithinases or phospholipases give microorganisms the ability to destroy human tissues by degrading lecithin (phosphatidylcholine) into phosphorylcholine and an insoluble diglyceride (Lazar and Balotescu, 2004; Holban et al., 2013).

By hydrolyzing starch, amylase causes the release of glucose, a carbon source useful for microorganisms in intracellular metabolic processes and, implicitly, in triggering bacterial pathogenic mechanisms; this is not significant for skin bacteria (Lazar and Balotescu, 2004; Chifiriuc and Lazar, 2011; Holban et al., 2013). It has been demonstrated that microorganisms lose their ability to multiply and express virulence factors in the absence of iron, an essential element in the development of these mechanisms of bacterial pathogenicity (Lazar and Balotescu, 2004; Chifiriuc and Lazar, 2011; Holban et al., 2013). When the extracellular environment is deficient in iron, to ensure the necessary intake, microorganisms express different virulence factors such as esculinase and bacterial hemolysins (Lazar and Balotescu, 2004; Chifiriuc and Lazar, 2011; Alina-Maria Holban et al., 2013). Esculetol has high affinity for iron and bacterial production of esculinase thus ensures the formation of iron stores necessary for the activation of microbial genes and the expression of other virulence factors (Lazar and Balotescu, 2004; Chifiriuc and Lazar, 2011; Holban et al., 2013). Hemolysins, by degrading hemoglobin, increase the concentration of iron in the environment and indirectly contribute to bacterial pathogenicity.

Caseinase, a proteolytic enzyme, intervenes in invasiveness by damaging the extracellular matrix of the connective tissue (Veis et al., 1997; Holban et al., 2013). Gelatinase is also an enzyme with a broad proteolytic spectrum (Holban et al., 2013; Ionescu et al., 2015).

In the conducted study, *S. aureus* strains predominantly expressed pore-forming toxins (lecithinase, lipase and hemolysins), necessary for systemic infectious dissemination. Moreover, MRSA strains compared to MSSA strains expressed, along with pore-forming toxins, also enzymes involved in the degradation of extracellular matrix proteins and cellular debris, such as caseinase and gelatinase.

Analysis of the phenotypic virulence profile for *P. aeruginosa* strains revealed an intense ability to express pore-forming toxins and exoenzymes. Compared to *S. aureus*, *P. aeruginosa* expressed in a higher proportion the proteases gelatinase and caseinase, involved in local invasiveness, but also hemolysins involved in dissemination. The strains belonging to the *Enterobacteriaceae* family produced virulence factors in a lower proportion compared to *S. aureus* and *P. aeruginosa* strains.

The strains isolated from venous ulcers expressed virulence factors involved in local invasiveness (caseinase, gelatinase), but also in the dissemination of infection (hemolysins). The virulence of MRSA strains isolated from venous ulcers was slightly higher compared to those from other sources. The strains of *P. aeruginosa* and those belonging to the *Enterobacteriaceae* family isolated from lower limb ulcers had a lower

virulence profile than chronic skin lesions of other etiology. The spectrum of soluble virulence factors of *P. aeruginosa* strains isolated from venous ulcers was different from that of strains isolated from other sources, suggesting the existence of some damaging factors that could modify the virulence profile of the microorganisms.

Patients with chronic venous ulcers had more frequent recurrences compared to patients suffering from leg ulcers of different etiology. A possible explanation is represented by the persistence of infections with bacterial strains that intensively develop biofilms, through the increased tolerance of these structures to antimicrobial substances or to the host's defense mechanisms.

MRSA strains possess various pathogenic mechanisms involved in infectious persistence, proving a high adaptability to the conditions in the external environment. Analysis of the phenotypic and genotypic virulence profile of MRSA strains isolated from delayed-healing wounds revealed several interesting results.

In comparison to other studied bacterial species, MRSA strains showed a more intense ability to adhere and aggregate to human cell substrate with the development of biofilms. The increased tolerance of biofilms to the action of antimicrobial molecules may further reduce the variety of effective therapeutic options in MRSA infections. This superior adherence and aggregation capacity may be explained by the detection in the vast majority of the studied strains of *clfA* and *clfB* genes, which encode adhesins or "clumping factors." These factors mediate the binding of *S. aureus* to fibrinogen and the initiation of infections from skin wounds, the adhesion of microorganisms to eukaryotic cells, but also to the surface of medical devices, thus causing nosocomial infections (Elgalai and Foster, 2003). These adhesins are included in the MSCRAMM (*microbial surface components recognizing adhesive matrix molecules*) category, along with protein A, fibronectin binding proteins (*FnbpA*, *FnbpB*), collagen binding protein and bone sialoprotein binding protein (Chifiriuc and Lazar, 2011).

MRSA strains were also more virulent compared to MSSA. Moreover, the virulence of MRSA strains isolated from venous ulcers was slightly higher compared to microorganisms from other sources.

Adhesion to fibronectin, mediated by the products of the *fnbA* and *fnbB* genes, is important in the adherence of bacteria to endothelial cells, thus being involved in the systemic dissemination of infections (Elgalai and Foster, 2003). Interestingly, the expression of the *fnbA* gene was associated with an increase in the degree of invasiveness of microorganisms and the transition from commensal colonization to systemic infection (Jenkins et al., 2015). In the present study, the *fnbA* gene was detected less frequently in MRSA strains isolated from venous ulcers, compared to other sources of isolation (arterial ulcers, mixed ulcers, chronic surgical wounds, chronic abscesses). This result could explain the rarity of severe systemic infectious events in patients with chronic venous disease.

The presence of a gene does not necessarily imply its phenotypic expression. For example, adhesion to fibronectin may vary, even if *fnbA* or *fnbB* are detected by PCR (Elgalai and Foster, 2003). Groups of researchers have observed that *S. aureus* strains show great diversity in their ability to adhere to different proteins (fibrinogen, fibronectin, collagen, laminin; Elgalai and Foster, 2003). Moreover, *S. aureus* expresses virulence factors differently, depending on the isolation source: the strains isolated by blood culture or from the peritoneal fluid being more virulent compared to those isolated from the tear secretion or from the nasal mucosa (Ionescu et al., 2015).

The *coag* gene encodes an extracellular protein, coagulase, which has two functions: fibrin clot formation by converting fibrinogen to fibrin and plasma coagulation by binding to prothrombin (Entenza et al., 2000). Thus, the microorganisms are protected from the action of the host's bactericidal molecules or from phagocytosis (Chifiriuc and Lazar, 2011). The belonging to the *aureus* species of *Staphylococccus* strains is confirmed by the coagulase production test. In the vast majority of the strains studied, the *coag* gene was detected (95.8%), involved in the protection of microorganisms from the action of the host's bactericidal molecules or from phagocytosis. It represents a third mechanism by which these microorganisms persist in chronic wounds, along with antibiotic resistance and biofilm tolerance to antibacterial agents.

EbpS encodes the elastin-binding protein, which gives microorganisms the property to adhere to elastin-rich tissues such as skin or lung tissue. Ionescu et al. (2015) reported the presence of the *Ebps* gene in 71.5% of a total of 144 MRSA strains isolated from nasal, ocular, skin wound secretions, peritoneal fluid or isolated by blood culture, and *clfA* and *clfB* genes were present in 99% of the strains. This gene was not detected in the strains studied in this research, suggesting the involvement of other mechanisms in the adhesion of MRSA isolated from chronic skin wounds to the cellular substrate.

This study is subject to several limitations that could be addressed in future research.

First of all, one limitation is represented by the heterogeneity in the study group. Patients diagnosed with chronic wounds usually suffer from cardiovascular diseases, obesity, diabetes mellitus, and other pathologies, with various degrees of severity, that may impact the prognosis of wound healing. One solution may be to include in future studies a larger number of patients randomly stratified by comorbidities and other individual factors.

Another limitation of the study is represented by the diagnosis heterogeneity in the control group. In future research we aim to restrict the inclusion criteria toward one particular disease in the control group (e.g., acute wound, surgical wound).

The follow-up may also show particular limitations. Although it was made at a standardized period of time, chronic wounds can be significantly impacted by multiple factors such as: compliance to treatment, hygiene conditions at home, management of comorbidities in an elderly population. Probably a better approach may be to reduce the follow-up to 1 week of hospitalization, where, under continuous medical supervision, a standard treatment can be kept more strictly and there can be obtained a better control of the comorbidities.

One of the main study limitations is represented by the use of traditional aerobic culturing techniques in the diagnosis of wound colonization or infection. Moreover, common bacteria, less virulent (coagulase-negative staphylococci) were excluded from the study. The study of commensal bacteria, as well as of the species isolated in anaerobic conditions may contribute to a superior knowledge on wound pathophysiology and on the complex host-microbiome interactions. In future research, in order to reveal the wounds microbiome diversity, we aim to perform both the aerobic and anaerobic microbiologic diagnosis. Moreover, depending on the funding, in order to reveal the diversity and architecture of mono- or poly-microbial biofilms, molecular tests may also be employed such as high-throughput sequencing, biofilm imaging with live/dead cells stains, fluorescence *in situ* hybridization (FISH) in combination with confocal laser scanning microscopy (CLSM).

Due to funding limitations, there was performed the genotypic characterization of the virulence profile only for MRSA strains, without including methicillin-sensitive staphylococci. In future studies we aim to expand the genotypic virulence assays for *Staphylococcus aureus* strains, as well as for other bacterial species.

5 Conclusion

The study revealed important differences regarding the clinical evolution and virulence profiles of microorganisms isolated from lower limb wounds, as well as between patients diagnosed with chronic venous ulcers and those with lesions of different etiology. The early identification of high-risk patients suffering of chronic venous ulcers, based on clinical, biological, and microbiological characteristics, may guide toward a personalized approach and, therefore, an optimized outcome. While most of the studies presented in the scientific literature focus on clinical prognostic markers of disease, there is still scarce information on the association of the virulence profile of microorganisms isolated from chronic venous ulcers with disease activity. Host-microbiome interplay and, implicitly, bacterial fitness, may lead to relevant phenotypic and genotypic traits of bacterial virulence, correlated with wound severity and response to therapy.

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 authors.

Ethics statement

The studies involving humans were approved by Ethics Commission of the "Elias" University Emergency Hospital, Bucharest. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MM: Funding acquisition, Investigation, Project administration, Visualization, Writing – original draft, Writing – review & editing. MP: Investigation, Writing – review & editing. AH: Investigation, Visualization, Writing – original draft, Writing – review & editing. IG-B: Investigation, Writing – review & editing. LP: Investigation, Writing – original draft. M-CC: Investigation, Writing – review & editing. CG: Writing – review & editing. CB: Investigation, Writing – review & editing. CC: Investigation, Writing – review & editing. VL: Funding acquisition, Project administration, Writing – original draft, 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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Supplementary material

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It's time to shed some light on the importance of fungi in neonatal intensive care units: what do we know about the neonatal mycobiome?

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The 21st century, thanks to the development of molecular methods, including DNA barcoding, using Sanger sequencing, and DNA metabarcoding, based on next-generation sequencing (NGS), is characterized by flourishing research on the human microbiome. Microbial dysbiosis is perceived as a new pathogenetic factor for neonatal diseases. Fungi are crucial, but neglected, components of the neonatal microbiome, which, despite their low abundance, significantly impact morbidity and mortality rates of premature infants hospitalized in Neonatal Intensive Care Units (NICUs). The neonatal mycobiome's composition and effect on health remain poorly studied research areas. Our knowledge about neonatal mycobiome, composed of limited genera, is mainly based on research on the bacterial microbiome. We presume it is influenced by clinical factors, including prematurity, antibiotic therapy, and type of delivery. Understanding these risk factors may be useful in prevention strategies against dysbiosis and invasive fungal infections. Despite the methodological challenges resulting from the biology of the fungal cell, this topic is an attractive area of research that may contribute to more effective treatment, especially of newborns from risk groups. In this mini review, we discuss the current state of knowledge, research gaps, study difficulties, and future research directions on the neonatal mycobiome, concerning potential future clinical applications.

KEYWORDS

neonatal mycobiome, neonatal intensive care unit, microbial dysbiosis, fungal microbiome, vertical transmission, prematurity, DNA barcoding, next generation sequencing

Introduction

The twenty-first century has witnessed intensive microbiome research in both animal and human studies. Bacteria and fungi became essential elements of the ecosystem, instead of playing just the role of pathogens (Shan et al., 2019). The researchers have worked on various aspects concerning the microbiome's development and function. It has become clear that the microbiome not only affects the infants' health but it is an important modulator of the adults' health in the long run. Microbial dysbiosis is correlated with abnormal development of the

immune and central nervous systems, as well as problems with digestion and metabolism (Yao et al., 2021). The infant microbiome depends on both environmental and genetic factors. Prenatal transmission of the microbiome depends on the maternal (endometrial, vaginal, gastrointestinal, and oral) microbiota, which is affected by genetics, diet, medications (especially antibiotics), infections, and stress. Moreover, there are microbiome differences resulting from the mode of delivery. During vaginal delivery, the newborns acquire bacteria specific to maternal vagina, such as *Lactobacillus*, *Senathia* spp., and *Prevotella*, while during cesarean section (C-section), the newborns are colonized by maternal skin bacteria, including *Staphylococcus*, *Propionibacterium,* and *Corynebacterium* (Yao et al., 2021). Another critical factor for infant microbial dysbiosis is gestational age at birth. It is connected with interruption of foeatal development during the third trimester, statistically higher rates of C-sections, and the medicalization of early stages of life (Arboleya et al., 2022). Finally, neonatal microbiome depends on human milk administration and kangaroo mother care. Human milk microbiota is characterized by a low bacterial load of high diversity, with the most predominant taxa, *Staphylococcus* and *Streptococcus* (Boudry et al., 2021). Moreover, the microbiome of breast milk is subject to dynamic changes not only during lactation but also during a single feeding session (Laursen et al., 2021). Skin-to-skin contact is associated with improved health outcomes for both the infant and the mother. As we know from one of the latest studies, it also affects microbiota composition in early infancy and alters its development, as measured by volatility and microbiota age (Eckermann et al., 2024).

Stop neglecting the fungi!

As mentioned earlier, most researchers have focused on the microbiome's bacterial components while ignoring the role of fungi in microbiome studies. Such a situation may contribute to bias in the research process (Ward et al., 2017). As far as we know, fungi cover 13% of the adult gut microbiome and comprise over 100 genera (Schei et al., 2017). The human mycobiome participates in many physiological processes occurring in the intestines (e.g., nutrients and vitamins absorption, micronutrient biosynthesis) and plays an essential role in the immunological processes, such as the presentation of antigens via pattern recognition receptors (James et al., 2020; Paul et al., 2020). Animal studies prove that gut mycobiome depends on diet and age. Mycobiome dysbiosis is observed in common diseases, like inflammatory bowel disease, colorectal adenomas, pouchitis, and diarrhea, as well as in rare conditions such as graft versus host disease and Rett syndrome. The correlation between fungal dysbiosis/atopic diseases is often studied in the pediatric population (Glatthardt et al., 2023). Data published by Boutin's research group revealed that a reduction of the relative abundance of the dominant age-associated fungal genus and/or an increase in fungal diversity in the first 3 months of life increases an infant's risk of asthma at the age of 5 years (Boutin et al., 2021). However, it is still unclear whether fungal dysbiosis is a consequence of the disease or if it plays a role in its etiology (Huseyin et al., 2017). That is why there is still a need to study how the human mycobiome evolves from birth to death.

The knowledge about pre- and postnatal transmission of the mycobiome is still limited; there are few studies on a similar subject. Figure 1 presents the timeline of selected milestones in mycobiome research. The first report, including a molecular analysis of the neonatal mycobiome, described 27 mother-infant pairs. The swabs were taken from the cheeks immediately after birth, then on the 1st and 3rd day of life, and 1 month after birth. The study confirmed vertical transmission of *Malassezia* (Nagata et al., 2012). Five years later, Schei et al. collected fecal samples from 298 mother-infant pairs. The maternal samples were collected at 35–38 gestational weeks of pregnancy and 3 months postpartum, while infant feces were gathered 10 days, 3 months, 1 year, and 2 years after birth. The mothers tended to have higher alpha and beta diversity in pregnancy than postpartum. The alpha diversity in the offspring seemed to increase steadily from birth, whereas the beta diversity was highest in 10-day-old infants. Interestingly, maternal fungal hosting made the offspring more susceptible to host fungi, which suggests that these mother-infant pairs share similar fungal hosting abilities. Because fungi are ubiquitous in the environment, they may originate from maternal genital tract and breastmilk, as well as contact with parental skin or hospital and home environment (Schei et al., 2017). As far as we know from the previous studies, breastmilk mycobiome is very diverse and consists of many fungal taxa (e.g., *Candida albicans, Candida parapsilosis, Cryptococcus neoformans, Saccharomyces cerevisiae, Candida glabrata*) differentiated mainly by geographic location and delivery mode (Boix-Amorós et al., 2019).

New insights into the pathogenicity of fungi

On the other hand, fungi play a significant role as infectious agents, especially among preterm and very low birth weight (<1,500 g) infants hospitalized in the neonatal intensive care units (NICUs). Invasive fungal infections are a leading cause of morbidity and mortality in preterm neonates due to the risk factors such as immunodeficiency, immaturity of the natural protective barriers, and invasive procedures (e.g., intravenous access and parenteral nutrition, endotracheal intubation). *Candida* species are the most common cause of fungal infections in neonates and the third cause of late-onset sepsis in NICU patients, determining invasive infection with a high burden of morbidity (including late neurodevelopmental sequelae) and mortality (Barton et al., 2017; Bennett, 2017). Although *Candida albicans* has historically been the most frequently isolated species, recently non-albicans *Candida* (NAC) have emerged as important opportunistic pathogens (Juyal et al., 2013). For this reason, fluconazole prophylaxis should be considered in high-risk infants, especially those admitted to NICUs with high rates of invasive candidiasis (Kilpatrick et al., 2022). What is more, antibiotic therapy, frequently used in NICUs, causes an iatrogenic mycobiome dysbiosis, which leads to an overgrowth of species belonging to the pathobionts (e.g., *C. albicans, C. metapsilosis, C. parapsilosis,* and *C. tropicalis*) and an increased risk of fungal infections (James et al., 2020).

A great threat for hospitalized patients is the emergence of multi-drug resistant pathogens such as *Candida auris*, which may cause candidemia associated with high mortality rates (Forsberg et al., 2019). This fungal pathogen was initially identified in 2009 from the external ear canal of a 70-year-old Japanese woman (Satoh et al., 2009). In the following years, it has spread worldwide, causing significant diagnostic and treatment challenges. Conventional

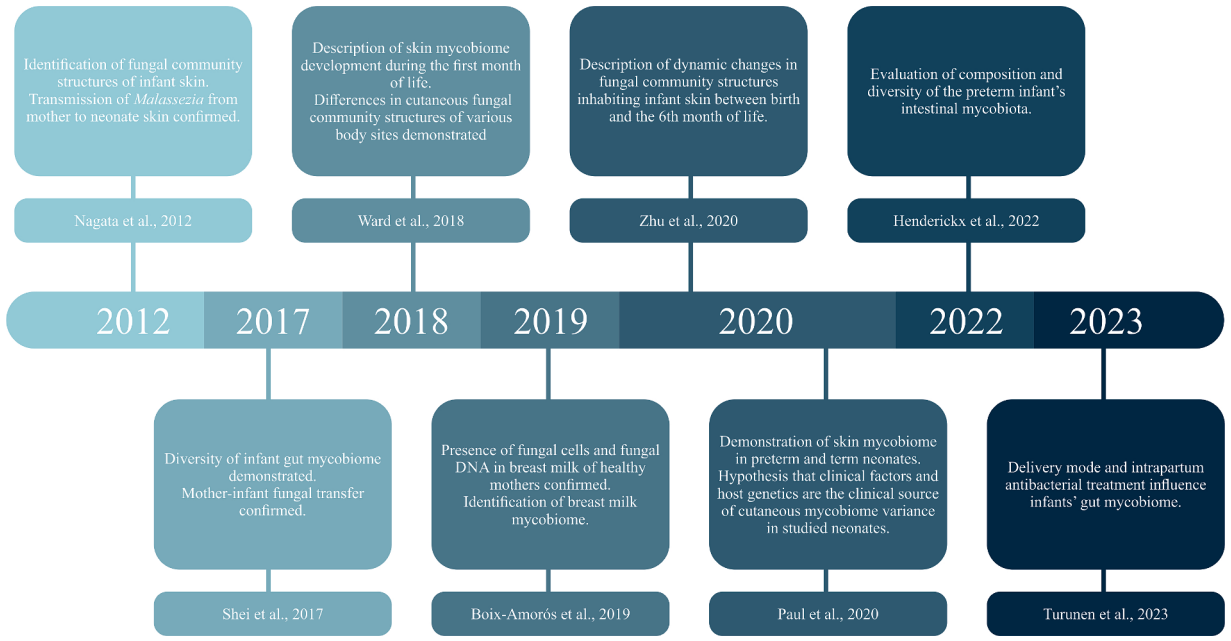

FIGURE 1
Timeline of selected milestones in mycobiome research.

microbiology methods do not allow it to be identified. Furthermore, *C. auris* is characterized by distinct mechanisms of antifungal resistance to azoles and amphotericin B. The highest morbidity rates occur in adults, children and newborns requiring chronic treatment in intensive care units (Alvarado-Socarras et al., 2021). Based on the case series, the neonatal population mortality rate can be estimated from 37.5 to 80% (Chandramati et al., 2020; Alvarado-Socarras et al., 2021; Ramya et al., 2021).

What is already known and current research gaps

Even though there are some previous studies concerning the neonatal mycobiome, they demonstrate mainly single-habitat descriptions. The best-studied area of research remains the intestine. The gut mycobiota was identified in most of the mothers and most of the offspring feces, supporting the thesis that fungi are an important element of the intestinal microbiome. The fungal species show a succession toward the maternal mycobiota as the child ages, with the most abundant *Debaryomyces hansenii* during breastfeeding and *Saccharomyces cerevisiae* after weaning (Schei et al., 2017; Turunen et al., 2023). The same issue was investigated by Henderickx et al., who showed that vaginally delivered infants present with the *Candida* genus. On the other hand, the *Malasseziomycetes* class and lower taxonomic levels are mainly characteristic for infants born through emergency C-section. Infants after vaginal and emergency C-section deliveries share fungi within the *Saccharomycetes* class but not for lower taxonomic levels. Infants delivered with a planned C-section are enriched in the *Microascales* and *Cladosporium* genera. Interestingly, while comparing newborns born prematurely with newborns born at term, *Candida* is the most abundant in both groups, but its load increases with gestational and postnatal age (Henderickx et al., 2022).

The second most frequently examined site is the skin. A previously mentioned study coordinated by Nagata revealed transmission of *Malassezia* spp. from mother to neonate (Nagata et al., 2012). Eight years later, a study on a larger group of children confirmed the dominance of the *Malassezia* genus and mainly *M.globosa* as the most abundant species. Skin sites and the subject's age were the major factors accounting for the diversity and composition of skin mycobiomes (Zhu et al., 2020). Assuming that the immaturity of the newborn's skin affects the skin mycobiome, a study on 15 full-term and 15 premature newborns was conducted. The most abundant genera in both preterm and term neonates were *Malassezia, Candida, Cladosporium, Fusarium*, and *Cryptococcus,* and the two most abundant species were *Malassezia restricta* and *Candida albicans.* Suprisingly no significant difference of relative abundances of these genera or species between preterm and term infants was found (Paul et al., 2020).

The study by Ward et al. shed a broader look at the newborn's mycobiome. The swabs from the skin, oral cavity, and anus of the infants were collected over the first month of life. Moreover, the maternal anal and vaginal swabs were taken after labor. The early infant mycobiome consisted of a few taxa (e.g., *C. parapsilosis* and *C. tropicalis*), across all infant body sites. Unlike previously cited studies, *Malassezia* accounted for only 2% of the relative abundance of skin mycobiome. *C. parapsilosis, C. tropicalis, C. orthopsilosis*, and *S. cerevisiae* were the most abundant and prevalent species in infant oral mycobiomes during the first month of life. Among the anal samples, *C. parapsilosis, C. tropicalis, C. albicans*, and *S. cerevisiae* were most common. Similarly, the maternal mycobiomes (vaginal and anal) were also dominated by a single taxon, mainly *C. albicans*. Due to the small group size, the study did not show statistically significant differences between the mode of delivery and the composition of the neonatal mycobiome. Regarding the authors' conclusions, there is a need to conduct further cross-sectional and longitudinal research with

larger cohorts, allowing to gather a more complete mycobiome description (Ward et al., 2018).

Vertical transmission is considered one of the most important factors when analyzing the newborn's mycobiome. Regarding research on the bacterial microbiome, studies on dyads (mother-infant pairs) make it possible to assess the impact of maternal health and mode of delivery on an infant's microbiome. In the study by Heisel et al. breastmilk was obtained from mothers when infants were 1 month old, and the infants' fecal samples were collected at 1 and 6 months of age. The most abundant and prevalent fungal species observed in breastmilk were *Paecilomyces dactylethromorphus*, *Fusarium equiseti, Malassezia restricta*, and *Candida albicans*. Similarly, the most abundant and prevalent fungal species in infant feces included *P. dactylethromorphus, M. restricta, C. albicans,* and *C. parapsilosis*. Moreover, bacterial-fungal correlations in breastmilk and infant feces were identified (Heisel et al., 2022). As far as we know, there is no data concerning kangaroo care, and its possible influence on neonatal mycobiome. Table 1 shows the most essential data about neonatal mycobiome and the gaps in knowledge that should be resolved in further studies. It emphasizes the need to conduct further research, considering not only mother-infant interactions but also associations regarding the non-fungal components of the microbiome.

Methodologies and challenges in neonatal mycobiome studies

The era of molecular research has opened new opportunities to determine the human mycobiome. Molecular methods, including DNA barcoding using Sanger sequencing and DNA metabarcoding based on next-generation sequencing (NGS) methods, have replaced morphology-based identification due to more accurate and unambiguous results. The molecular approaches focus on DNA sequences (markers), which discriminate the fungal taxa. The official marker of fungal identification is the internal transcribed region (ITS), located in nuclear ribosomal RNA, which exhibits high genetic variations; thus, it is useful for fungal recognition (Raja et al., 2017). However, in the case of highly speciose genera of fungi (e.g., *Fusarium*, *Penicillium*, *Aspergillus*), the ITS sequence is insufficient to differentiate the species, so in those cases, additional DNA fragments are included in analyses (e.g., translation elongation factor, beta-tubulin, actin). DNA barcoding requires *in vitro* cultivation of fungal strains, which allows for detailed analyses to identify the fungal species; however, in this approach, the information about slow-growing and biotrophic fungi is lost.

On the contrary, DNA metabarcoding is culture-independent, so it can be performed directly from the tissue/body fluids. However, the identification with the use of most currently used NGS approach is limited to short ITS fragments (<500 base pair [bp]). To overcome the issue, we recommend using both DNA barcoding and metabarcoding or/and using the NGS Platform to obtain longer reads, providing higher taxonomic resolution (e.g., PacBio, Oxford Nanopore). Using this NGS platform, it is possible to obtain the sequences of the whole fungal rRNA operon of approximately 5,5 kbp in length (Lu et al., 2023).

The DNA extraction from fungal cells is challenging. Fungi, unlike bacteria, have a cell wall that is usually composed of, ß-1,3-glucan, ß-1,6-glucan, mannans, several glycoproteins and can also contain melanin or a rodlet layer (Thielemann et al., 2022). Thus, an essential step for fungal DNA extraction is efficient cell wall lysis by mechanic methods, like repeated beat-beating, followed by enzymatic cell lysis. Importantly, as with bacterial studies, standardization protocol for DNA isolation from fungal cells in human mycological studies is urgently needed. A sample-specific DNA extraction procedure with the possibility of its validation is recommended (Tiew et al., 2020). A wide range of ready-to-use techniques have been applied; for instance, the ZymoBIOMICS DNA Kit (Zymo Research, United States) was successfully used for human swab samples (Burton et al., 2022) and InviMag stool DNA kit (Stratec Molecular, Germany) preceded by mechanical and chemical cell lysis were adopted for breast milk samples (Boix-Amorós et al., 2019).

The molecular methods provide tremendous amounts of data; thus, the precise recruiting of the study groups is crucial for interpreting the obtained results. Considering that the human's mycobiome composition can be affected by various factors, the specimen collection, sample processing, and the patient's description must be prepared according to standardized procedures.

The most extensive and recommended database used for fungi taxonomy assignment is UNITE, based on data from the International Nucleotide Sequence Database Consortium (Nilsson et al., 2019). The UNITE contains 8,381,941 ITS sequences grouped into 223,659 fungal Species Hypotheses with digital object identifiers (DOIs) at a 1.5% threshold (as of 14 November 2023). The indicated database also contains non-fungal sequences that help distinguish fungi from other eukaryotes. Furthermore, it is constantly developed and allows for users' data submission. However, it is essential to remember that the estimated number of fungal species is between 2.2 and 3.8 million, but much fewer are named and described (Hawksworth and Lücking, 2017). Thus, it might happen that the studied fungal community will not be entirely determined despite using large databases.

Potential future developments in the field

The human microbiome is perceived as a primary target of future personalized medicine. Finding potential interactions between microbial dysbiosis and diseases may contribute to more effective treatment and disease prevention (Berg et al., 2020). Currently, the knowledge about the influence of probiotics on the neonatal mycobiome is poor. According to our knowledge the only study that assessed the impact of probiotics on the infants' mycobiome is the previously mentioned research conducted by Schei et al. Study participants were enrolled into a population-based, randomized, placebo-controlled, and double-blinded trial on probiotics. The authors investigated how consuming milk with probiotic bacteria by pregnant and breastfeeding women affects the infants' gut mycobiome. No statistically significant effects of maternal probiotic intake on offspring gut mycobiome were observed. Due to challenges in mycobiome research, the authors emphasize the need for further research (Schei et al., 2017, 2020).

Consequently, to the increasing rate of C-section, an interesting area of research remains vaginal seeding. During the procedure, a sterile gauze pad is placed in the mother's vagina before the planned

TABLE 1 The most essential data about neonatal mycobiome and the gaps in knowledge.

What do we know?	Gaps in knowledge
Structure of fungal communities residing on infants' skin (Nagata et al., 2012), in the gut (Schei et al., 2017), and in breast milk (Boix-Amorós et al., 2019).	• Lack of functional studies of identified fungal communities. What is the role of identified fungi in healthy newborns? • Knowledge about the entire mycobiome (altogether: skin, oral cavity, gut, breast milk) of mother-newborn pairs and its dynamic during the first few weeks after birth. • Identification of beneficial fungal species and/or positive for human health fungal communities.
Fungi are transmitted from mother to offspring (Nagata et al., 2012; Shei et al., 2017).	• Whether all genera of fungi associated with the mother can transfer to her offspring? • Which pathway /and what type of transmission are predominant? • Which factors can alter the transmission?
The infant mycobiome is altered by: • delivery mode (Boix-Amorós et al., 2019; Paul et al., 2020; Heisel et al., 2022; Turunen et al., 2023), • geographic location (Boix-Amorós et al., 2019), • antibiotics exposure (Paul et al., 2020; Heisel et al., 2022; Turunen et al., 2023) • host genetics – single nucleotide polymorphisms (SNPs) in genes mediating the response for fungal and bacterial expansion (Paul et al., 2020), • diet (Paul et al., 2020), • neonatal intensive care unit (NICU) environment (Paul et al., 2020).	• Complete understanding of the impact of the environment on newborn mycobiome. • How can the newborn mycobiome be modulated? o Role of breastfeeding o Kangaroo mother care • What conditions favor the colonization of neonates by beneficial fungi? • Do other genetic or epigenetic host components shape the mycobiome?
Correlation between bacterial and fungal taxa abundance in fecal and breast milk samples (Heisel et al., 2022).	• Knowledge about the interaction and relationship between bacteria and fungi associated with neonate tissues and their role in health modulation.

C-section. After delivery, the neonatal team wipes the baby's mouth, face, and entire body with the gauze mentioned above. Scientists are investigating the effectiveness of this method in the transmission of the maternal reproductive tract flora to a newborn born by C-section. The first double-blind, randomized, placebo-controlled trial revealed that vaginal seeding significantly increases the bacterial load in the skin but not in the transitional stool and, when compared with the control group, causes a significant reduction in alpha diversity in the skin and transitional stool. The authors emphasize the need for further research to determine if these effects persist over time and whether they bring any health benefits (Mueller et al., 2023).

Another promising concept is maternal fecal microbiota transplantation. During the procedure, a processed maternal fecal sample, mixed with the mother's milk (obtained before the C-section or, if needed, pasteurized bank milk), is administered in the first feeding of the infant within 2 h of birth. It has been shown that this procedure is more effective than vaginal seeding in the context of restoring the normal microbiome pattern in newborns born by C-section. This indicates a much more significant impact of fecal bacteria on the proper development of the newborn's microbiome after birth (Korpela et al., 2020). Even though current research still lacks data to support this discovery in terms of mycobiome.

Considering differences resulting from gestational age and the occurrence of diseases typical for prematurely born newborns, like necrotizing enterocolitis (NEC), makes it possible to suspect the impact of mycobiome disorders on increased morbidity and mortality in this group of patients. Moreover, identifying the possible correlations between dysbiosis and complications related to premature birth may enable applying preventive strategies. NEC remains one of the most important and unresolved problems in neonatology, etiology of which is associated with intestinal dysbiosis. While no particular pathogen has been linked with NEC development, a general reduction in bacterial diversity and an increase in pathobiont abundance has been noted preceding disease onset. Currently, we do not have studies that would indicate the role of fungi in the development of NEC in newborns (Wilson et al., 2023). Research on mouse models suggests the potentially beneficial effects of β-glucan in preventing the development of NEC. B glucan is a kind of bioactive polysaccharide obtained from yeast, which, among many health-promoting activities, is also credited with a regulating effect on the microbiome. Despite promising results indicating the effect of β-glucan administration on intestinal injury of NEC mice, further research is needed to determine whether a similar phenomenon is observed in newborns (Zhang et al., 2023).

Conclusion

The neonatal mycobiome remains neglected despite intensive research on the human microbiome. Molecular methods open up new research possibilities, which may bring us closer to personalized medicine in this susceptible group of patients, especially those born prematurely. Analysis of current studies leaves many research gaps and opportunities for potential future developments, such as the impact of vertical transmission on the development of the infant's mycobiome and the role of probiotics and microbiota transplant in restoring microbial balance. Moreover, it is crucial to clarify the role of fungi as potential factors in the etipathogenesis of prematurity-related diseases, such as necrotizing enterocolitis.

To conclude, it is recommended that further cross-sectional studies on larger cohorts, incorporating the maternal mycobiome status, should be conducted.

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DW: Conceptualization, Investigation, Visualisation, Writing – original draft. SS: Methodology, Writing – original draft. KW-S: 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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