

Developments in non-antibiotic antimicrobials in the era of drug-resistant bacteria

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Developments in non-antibiotic antimicrobials in the era of drug-resistant bacteria

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Antimicrobial activity of α -mangostin against *Staphylococcus* species from companion animals *in vitro* and therapeutic potential of α -mangostin in skin diseases caused by *S. pseudintermedius*

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Antimicrobial resistance in *Staphylococcus* species from companion animals is becoming increasingly prevalent worldwide. *S. pseudintermedius* is a leading cause of skin infections in companion animals. α -mangostin (α -MG) exhibits various pharmacological activities, including antimicrobial activity against G (+) bacteria. This study investigated the antimicrobial activity of α -MG against clinical isolates of *Staphylococcus* species from companion animals and assessed the therapeutic potential of α -MG in skin diseases induced by *S. pseudintermedius* in a murine model. Furthermore, the action mechanisms of α -MG against *S. pseudintermedius* were investigated. α -MG exhibited antimicrobial activity against clinical isolates of five different *Staphylococcus* species from skin diseases of companion animals *in vitro*, but not G (-) bacteria. α -MG specifically interacted with the major histocompatibility complex II analogous protein (MAP) domain-containing protein located in the cytoplasmic membrane of *S. pseudintermedius* via hydroxyl groups at C-3 and C-6. Pretreatment of *S. pseudintermedius* with anti-MAP domain-containing protein polyclonal serum significantly reduced the antimicrobial activity of α -MG. The sub-minimum inhibitory concentration of α -MG differentially regulated 194 genes, especially metabolic pathway and virulence determinants, in *S. pseudintermedius*. α -MG in pluronic lecithin organogel significantly reduced the bacterial number, partially restored the epidermal barrier, and suppressed the expression of cytokine genes associated with pro-inflammatory, Th1, Th2, and Th17 in skin lesions induced by *S. pseudintermedius* in a murine model. Thus, α -MG is a potential therapeutic candidate for treating skin diseases caused by *Staphylococcus* species in companion animals.

KEYWORDS

Staphylococcus species, companion animals, α -Mangostin, antimicrobial activity, MAP domain-containing protein

Introduction

Antimicrobial resistance (AMR) is a major threat to public health and veterinary medicine (World Health Organization, 2014; World Organisation for Animal Health, 2016). AMR in bacterial pathogens derived from livestock is increasing due to the misuse and overuse of antimicrobial agents for treatment, metaphylaxis, and growth promotion (Van Boeckel et al., 2015). In addition, AMR in bacterial pathogens from companion animals is an emerging problem that increases the risk to public health because antimicrobial agents important in human medicine are commonly used to treat bacterial infections in companion animals (Johnston, 1998; Joosten et al., 2020). Livestock and companion animals are potential reservoirs of resistant bacteria and AMR genes (Joosten et al., 2020; Ibekwe et al., 2023). AMR genes or resistant bacteria can be transferred between companion animals and humans by close contact, food chain, or environment (Bennani et al., 2020).

The most common reason to visit veterinary hospitals for disease treatment is skin and subcutaneous infections such as dermatitis and pyoderma in Korea (Rural Development Administration of Korea, 2018). *Staphylococcus* species, including *S. pseudintermedius*, *S. schleiferi*, and *S. felis*, are most commonly isolated from skin lesions of companion animals (Devriese et al., 2009; Palomino-Farfan et al., 2021; Cavana et al., 2023). *S. pseudintermedius* is the leading cause of skin, ear, and postoperative bacterial infections in dogs and cats (Devriese et al., 2009; Bannoehr and Guardabassi, 2012; Pompilio et al., 2015). Pyoderma caused by *Staphylococcus* species in companion animals is commonly treated with antimicrobial agents. However, multidrug resistance was found in 75% of *S. pseudintermedius* isolates and 39% of *S. schleiferi* isolates from companion dogs and cats (Animal and Plant Quarantine Agency, 2020). Notably, resistance to methicillin was found in 40% and 27% of *S. schleiferi* and *S. pseudintermedius* isolates, respectively (Animal and Plant Quarantine Agency, 2020). Regarding the prevalence of AMR in *Staphylococcus* species from skin lesions of companion animals, it is necessary to develop antibiotic alternatives to control bacterial skin infections in companion dogs and cats.

Natural products are an important source of antimicrobial agents or serve as templates for synthetic drugs. A wide range of structurally unique secondary metabolites from plants is also important for developing new antimicrobial agents (Zaynab et al., 2018). Mangosteen (*Garcinia mangostana* Linn) is a tropical fruit tree cultivated in Southeast Asia for centuries. Mangosteen pericarps have been used as a traditional medicine for treating cystitis, diarrhea, abdominal pain, eczema, fever, itching, and other skin diseases (Pedraza-Chaverri et al., 2008; Zaynab et al., 2018; Gunter et al., 2020). The bioactive secondary metabolites of mangosteen are xanthone derivatives that possess a potent pharmacological activity (Jung et al., 2006; Akao et al., 2008; Gunter et al., 2020; Liu et al., 2022). α -mangostin (α -MG) is a major xanthone derivative that exhibits broad-spectrum pharmaceutical activities, including antibacterial, antifungal, antiparasitic, anti-inflammatory, antitumor, antidiabetic, antioxidant, and antiobesity (Iinuma et al., 1996; Ibrahim et al.,

2016). α -MG has demonstrated antimicrobial activity against G (+) bacteria such as *S. aureus*, *S. epidermidis*, *Streptococcus pyogenes*, *S. mutans*, and vancomycin-resistant enterococci (Sakagami et al., 2005; Nguyen et al., 2014; Sivarajani et al., 2017; Felix et al., 2022). α -MG interacts with the cytoplasmic membrane of *S. aureus* and disrupts the cytoplasmic membrane integrity, resulting in a rapid bactericidal action (Koh et al., 2013; Felix et al., 2022), but the exact action mechanisms of α -MG in killing *Staphylococcus* species have not been characterized yet. Moreover, the antimicrobial activity of α -MG against *Staphylococcus* species commonly isolated from companion animals has not been determined. This study was aimed to investigate the antimicrobial activity of α -MG against clinical isolates of *Staphylococcus* species from skin diseases of companion dogs and cats and elucidate the action mechanisms of α -MG in killing *S. pseudintermedius*. Finally, the therapeutic potential of α -MG in skin diseases of companion dogs and cats induced by *S. pseudintermedius* was assessed in a murine model.

Materials and methods

Bacterial strains

Ten reference bacterial strains, including eight different *Staphylococcus* species, *Escherichia coli*, and *Pseudomonas aeruginosa*, were used in this study (Table 1). Seven and three reference strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and Korean Collection for Type Cultures (KCTC; Daejeon, Korea), respectively. A total of 277 clinical isolates, including *S. pseudintermedius* (n = 80), *S. schleiferi* (n = 64), *S. felis* (n = 51), *S. epidermidis* (n = 27), *S. aureus* (n = 19), *E. coli* (n = 20), and *P. aeruginosa* (n = 16), from skin diseases of companion dogs (n = 206) and cats (n = 71) were obtained from the Animal and Plant Quarantine Agency (Gimcheon, Korea).

Antimicrobial susceptibility testing

The broth microdilution method was used to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of α -MG. α -MG was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 25 mg/ml and used as stock solution. The agar dilution method was also used to determine the MICs of α -MG. The antimicrobial activity testing of α -MG was performed by the guidelines of Clinical Laboratory Standards Institute (CLSI, 2020). Serial two-fold dilutions of α -MG were prepared in Mueller-Hinton agar or Mueller-Hinton broth (MHB; Difco, Detroit, MI, USA).

Time-kill kinetics assay

Three bacterial strains (*S. felis* ATCC 49168, *S. pseudintermedius* ATCC 49051, and *S. schleiferi* ATCC 43808)

TABLE 1 *In vitro* antimicrobial activity of α -MG against *Staphylococcus* species and G (-) bacterial strains.

Bacteria	MIC (μ g/ml)		MBC (μ g/ml)
	Agar dilution	Broth microdilution	
<i>S. aureus</i> ATCC 29213	4	2	8
<i>S. carprae</i> KCTC 3583	2	2	4
<i>S. epidermidis</i> ATCC 12228	1	1	4
<i>S. felis</i> ATCC 49168	2	1	4
<i>S. intermedius</i> KCTC 3344	2	1	4
<i>S. pseudintermedius</i> ATCC 49051	2	1	2
<i>S. saprophyticus</i> KCTC 3345	2	1	2
<i>S. schleiferi</i> ATCC 43808	1	1	1
<i>E. coli</i> ATCC 25922	>64	>64	>64
<i>P. aeruginosa</i> ATCC 27853	>64	>64	>64

*MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

were grown overnight in MHB at 37°C. Bacterial cultures were diluted to 1:100 in 10 ml MHB and 200 μ l bacterial suspension was inoculated in each well in 96-well plates containing MHB with two-fold serially diluted α -MG (1/4 $\times$ to 1 $\times$ MIC). The plates were incubated at 37°C with shaking (150 rpm) for 18 h. The optical density at 600 nm (OD₆₀₀) was measured every 1 h using a microplate reader (Molecular Devices, San Jose, CA, USA).

Field emission-scanning electron microscopy

Three bacterial strains (*S. felis* ATCC 49168, *S. pseudintermedius* ATCC 49051, and *S. schleiferi* ATCC 43808) were grown overnight in lysogeny broth (LB) at 37°C. Each bacterial culture (10 μ l) was inoculated in LB containing different α -MG concentrations (1/4 $\times$, 1/2 $\times$, 1 $\times$, 2 $\times$, and 4 $\times$ MICs) in six-well plates with poly-L-lysine-coated cover glass (Sigma-Aldrich), and the plates were incubated at 37°C for 2 h. The cover glasses were removed from the plates and washed twice with phosphate-buffered saline (PBS). The samples were serially dehydrated with 20%, 50%, and 70% ethanol for 10 min, and 100% ethanol for 30 min. The samples were coated with platinum, and ultrastructural changes of bacteria were observed using FE-SEM (Hitachi SU-8220; Hitachi, Japan).

Biotinylation of α -MG

α -MG was purchased from Combi-blocks (San Diego, CA, USA). For biotinylation of α -MG, biotin (82.4 mg, 0.33 mM) and hexafluorophosphate azabenzotriazole tetramethyl uranium (128.3 mg, 0.33 mM) in dimethylformamide (5 ml) and triethylamine (68.3 mg, 0.67 mM) were added in the stirred solution of α -MG (92.3 mg, 0.22 mM) at 0°C and stirred for 16 h at room temperature.

The reaction mixture was diluted with CH₂Cl₂ (25 ml), and the solution was washed twice with distilled water (20 ml) and once with brine (20 ml). The sample solution was dried over anhydrous Na₂SO₄ and evaporated. The samples were purified by column chromatography (silica gel, 2%-5% methyl alcohol in CH₂Cl₂) to make the tile compound (100 mg, 51% yield) a pale-yellow solid. ¹H nuclear magnetic resonance (400 MHz, CD₃CN): δ _H 13.80 (s, 1H), 13.39 (s, 1H), 7.16 (s, 1H), 6.84 (s, 1H), 6.64 (s, 1H), 6.38 (s, 1H), 5.38 (d, *J* = 18.5 Hz, 3H), 5.23 (d, *J* = 1.4 Hz, 2H), 5.12 (t, *J* = 7.0 Hz, 1H), 4.53-4.38 (m, 2H), 4.35-4.20 (m, 2H), 4.16-4.02 (m, 3H), 3.77 (d, *J* = 7.9 Hz, 3H), 3.42-3.12 (m, 4H), 2.91 (ddd, *J* = 14.4, 7.2, 5.5 Hz, 2H), 2.68 (dt, *J* = 15.2, 7.1 Hz, 4H), 1.84-1.74 (m, 12H), 1.73-1.63 (m, 10H) ppm; mass spectrometry (MS)-electrospray ionization: *m/z* [M-H]⁺ calculated for C₃₄H₃₉N₂O₈S 635.24; found 635.26. Chemical structure of biotinylated α -MG was depicted in [Figure S1](#).

Protein pull-down assay

The protein pull-down assay was conducted using the Biotinylated Protein Interaction Pull-Down Kit (Thermo Fisher Scientific, Waltham, MA, USA). *S. pseudintermedius* ATCC 49051 was cultured overnight in LB at 37°C. After centrifugation of bacterial culture, the pellets were washed with PBS and suspended in buffer A [20 mM Tris-HCl (pH 7.5), 0.5 M NaCl]. Bacteria were lysed by sonication (Branson Sonifier 450; Branson Ultrasonics, Danbury, CT, USA). Biotinylated α -MG (20 mM) was added to streptavidin containing gel and incubated at 4°C for 2 h with gentle shaking. After removing the free biotinylated α -MG, the gel was blocked with a biotin solution for 5 min. Bacterial lysates were added to the gels and incubated at 4°C for 2 h with gentle shaking. The gel was washed twice with 0.25 M NaCl buffer, and bacterial proteins bound to biotinylated α -MG were obtained using an elution buffer.

Liquid chromatography-MS

Protein samples were denatured by boiling at 95°C for 10 min and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stained using a PowerStain Silver Stain Kit (Elpis, Daejeon, Korea). The specific protein bands were excised for subsequent gel digestion and analysis. Digested tryptic peptide samples were applied to 100 $\mu\text{m} \times 2$ cm nanoViper trap columns (Thermo Fisher Scientific) to enrich the peptides and remove chemical contaminants. Concentrated tryptic peptides from the trap column were loaded to the 15 cm $\times$ 75 μm nanoViper analysis column (Thermo Fisher Scientific) at 300 nl/min flow rate. Peptides were eluted using a gradient from 0 to 65% acetonitrile for 80 min. All MS and MS/MS spectra were acquired in a data dependent mode using a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific). Each full MS (m/z range of 300 - 2,000) scan was followed by three MS/MS scans of the most abundant precursor ions in MS spectra. MS/MS spectra were analyzed by MASCOT version 2.7 (Matrix Science, Boston, MA, USA) using protein sequences from the genome of *S. pseudintermedius* (NZ_CP065921.1) downloaded from the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>).

Production of recombinant major histocompatibility complex II analogous protein domain-containing proteins and mouse anti-MAP domain-containing protein polyclonal serum

The gene encoding the full-length MAP domain-containing protein (QIW05685.1 in *S. pseudintermedius* VTH737) was amplified by polymerase chain reaction (PCR) using the genomic DNA from *S. pseudintermedius* ATCC 49051 as a template. The specific primers (5'-GGG CGG CGG TGG TGG CGG CAT GAA AGC AAA AAA ATT ATT AGC TAC AGG-3' and 5'-GTT CTT CTC CTT TGC GCC CTA TTA TTT CGC ACT TAC CAC TTC AAT-3') were designed for ligation-independent cloning. After treating T4 DNA polymerase (New England Biolabs, Ipswich, MA, USA), the amplicons were inserted into pB3 plasmids derived from the pET21a plasmid (Figure S2A). The construction was confirmed by DNA sequencing analysis. The constructed plasmids were transformed into *E. coli* BL21 (DE3) cells and grown in LB to reach an OD₆₀₀ of 0.4 - 0.5. The expression of recombinant proteins was induced with 0.2 mM isopropyl β -D-1-thiogalactopyranoside overnight at 18°C. The recombinant proteins were purified in a His-Trap HP column (GE Healthcare, Chicago, IL, USA). The recombinant proteins were purified by size exclusion chromatography (Figure S2B). The purified protein fractions were separated by 10% SDS-PAGE and stained with Coomassie blue G-250 (iNtRON Biotechnology, Seongnam, Korea) (Figure S2C). BALB/c mice (6-weeks old) were used to produce anti-MAP-domain containing protein polyclonal serum. The purified recombinant proteins (250 μg) were emulsified with the same volume of Freud's complete adjuvant (FCA; Sigma-Aldrich) for

the first injection of antigens and 50 μg proteins were emulsified with the same volume of Freud's incomplete adjuvant (FIA; Sigma-Aldrich) for the boosting shot. Blood was obtained by retroorbital bleeding before immunization to prepare the pre-immune sera. On the first day, proteins emulsified with FCA were subcutaneously delivered to four sites of the mouse back. For boosting, proteins emulsified with FIA were injected similarly on the 21st and 42nd days. Whole blood was harvested on the 50th day.

Bacterial killing in serum

S. pseudintermedius ATCC 49051 was cultured in trypticase soy agar (Difco) overnight at 37°C and resuspended in PBS. Bacteria [10^5 - 10^6 colony forming units (CFUs)/ml] were added to the antiserum immunized with recombinant MAP domain-containing protein prepared at a ratio of 1:500 and incubated at 37°C for 20 min. Different α -MG concentrations (1 $\times$ to 4 $\times$ MICs) were added to the tubes. The samples were collected at the indicated time points (0, 5, 10, and 20 min), and the CFU was determined. The experiments were performed in three independent experiments.

Localization of MAP-domain containing protein

Whole cell lysates, cell wall-associated proteins, and membrane vesicles (MVs) were prepared from *S. pseudintermedius* ATCC 49051. To prepare whole cell lysates, bacteria were cultured in LB at 37°C for 16 h and bacterial cultures were centrifuged at 6,000 g at 4°C for 15 min. Bacteria were lysed by sonication. To prepare cell wall-associated proteins, bacteria were cultured in LB at 37°C for 16 h. After centrifugation, bacterial cells were placed on chilled ice for 5 min and resuspended in 5 ml TE buffer [50 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (pH 8.0), and 1 mM phenylmethyl sulfonyl fluoride]. After centrifugation, bacterial cells were resuspended in 1.15 ml mutanolysin mixture [1 ml TES buffer (20% w/v sucrose in TE buffer), 100 μl lysozyme (100 mg/ml), and 50 μl mutanolysin (5,000 units/ml)] and incubated at 37°C for 2 h with shaking. The samples were centrifuged at 14,000 g for 5 min. MVs were prepared from bacterial culture supernatants as previously described (Jun et al., 2017). Bacteria were cultured in 1 L LB at 37°C with shaking to reach OD₆₀₀ of 0.5 and MVs were purified. Purified MVs were checked for sterility on blood agar plates. Protein concentrations of the three samples were measured using the BCA assay (Thermo Fisher Scientific). Proteins (4 μg) were separated by 10% SDS-PAGE and electro transferred to a polyvinylidene difluoride membrane (Thermo Fisher Scientific). The membrane was blocked with a 5% skim milk solution in TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, and 0.01% Tween-20) for 2 h at room temperature. The membranes were treated with polyclonal antisera diluted with 1:1000 for 1 h and washed three times with TBS-T buffer. The membranes were incubated with secondary goat anti-mouse IgG-horseradish peroxidase-conjugated antibody diluted with 1:2000 (Ab Frontier, Seoul, Korea) for 1 h. The membrane was developed using SuperSignalTM West Pico Plus chemiluminescent substrates (Thermo Fisher Scientific).

RNA extraction and transcriptome analysis

Transcriptome analysis of *S. pseudintermedius* treated with α -MG was performed as previously described with some modifications (Sivaranjani et al., 2019). *S. pseudintermedius* ATCC 49051 was grown to mid-log phase ($\sim 1.0 \times 10^8$ CFU/ml) and treated with $0.7 \times \text{MIC}$ ($0.7 \mu\text{g/ml}$) of α -MG for 10 min. Bacteria were treated with 0.003% DMSO as a control. Total RNAs were prepared using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The residual genomic DNA was digested by RNase-free DNase (Qiagen, Hilden, Germany). The structural integrity and quality of extracted RNAs were verified by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA USA). Two biological replicates were used to produce RNA samples for sequencing. To construct strand-specific libraries, rRNA was depleted using the Ribo-Zero rRNA Removal Kit (Illumina, San Diego, CA, USA). Libraries were constructed using a TruSeq Stranded Total RNA Sample Preparation Kit (Illumina) according to the manufacturer's instructions. Briefly, rRNA-depleted RNAs were fragmented into short fragments (200 - 500 nt) using a Covaris S220 ultrasonicator (Woburn, MA, USA), and first-strand cDNA was synthesized from fragments using random hexamer primers and reverse transcriptase, followed by the synthesis of the second strand with dUTP substituted for dTTP. Ends of short fragments were processed by adding a single adenine after purification and connected with adapters. Finally, the second strand was degraded using uracil-N-glycosylase and PCR-amplified for eight cycles to construct strand-specific libraries. The constructed library was amplified with phi29 to make a DNA nanowall (DNB) with >300 copies of one molecule. DNBs were loaded into the patterned nanoarray, and paired-end 100 bases reads were generated via sequencing by combinatorial Probe-Ancor Synthesis using a DNBSEQ-T7 sequencing machine (BGI, Shenzhen, Guangdong, China). The quality of the raw reads (FASTQ) was evaluated using FastQC version 0.11.5 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), and the dirty reads were removed before downstream analysis to decrease data noise. The reads were subjected to standard quality control criteria according to the following parameters: (1) trimming and cleaning reads that aligned to primers and/or adaptors, (2) reads with >50% of low-quality bases (quality value ≤ 5) in one read, and (3) reads with >10% unknown bases (N bases). After filtering, the remaining reads were called clean reads and stored in FASTQ format. All high-quality and clean reads were subjected to be mapped to the NCBI reference genome *S. pseudintermedius* ASM1612671v1 using HISAT to obtain the BAM file. BAM files were sorted and indexed with Samtools, and the number of reads matching each gene in *S. pseudintermedius* was counted with HTSeq-count (Anders et al., 2015; Kim et al., 2015). Read-count data were filtered (rowMax <1) and normalized using DESeq2 (version 1.26) and R packages (version 3.6.1) according to the protocol. Fold-change ratio and Wald test were performed between the control and test groups using DESeq2. Differentially expressed genes (DEGs) were considered when the fold-change ratio ≥ 2 or ≤ 0.5 and the Wald test $q < 0.05$. Data visualization such as scatterplot, boxplot, volcano plot, and hit map was plotted using the R platform. The Kyoto Encyclopedia of Genes and Genomes (KEGG)

pathway assignment was performed to determine the function of differentially regulated genes. Hypothetical proteins were analyzed for putative functions using protein BLAST to search for Pfam (protein families). Signal peptides and the location of cleavage sites in proteins were predicted using SignalP-5.0 (<https://services.healthtech.dtu.dk/service.php?SignalP-5.0>). Subcellular localization was predicted using PSORTb (<https://psort.org/psortb>). Three dimensional structure of protein was predicted using the AlphaFold program (<http://alphafold.ebi.ac.uk>).

Quantitative real-time PCR

Total RNA from *S. pseudintermedius* ATCC 49051 was isolated as described above, and cDNA was synthesized using the M-MLV cDNA synthesis kit (Enzynomics, Daejeon, Korea). qPCR was performed using an ABI Step One Plus Real-Time System (Applied Biosystems, Waltham, MA, USA). TOPreal™ q-PCR 2X PreMix (SYBR Green with high ROX; Enzynomics) was used. Total RNA was also isolated from the dissected left ears of mice in *in vivo* experiments. The primers used to validate qPCR and the expression of cytokine genes are listed in [Supplementary Table S1](#). The expression level was standardized against constitutively expressed 16S rRNA and glyceraldehyde 3-phosphate dehydrogenase expression levels and quantified using the $2^{-\Delta\Delta C_t}$ method. qPCR was performed in three independent experiments.

Cell culture and cytotoxicity assay

Canine progenitor epidermal keratinocytes (CPEK) were purchased from CELLnTEC Advanced Cell System (Bern, Switzerland). CPEK cells were cultured in CnT-09 medium (CELLnTEC Advanced Cell System) at 37°C in a humidified 5% CO₂ atmosphere. Cells were seeded in a 96-well plate at a density of 8.0×10^3 cells/well. Cells were treated with different α -MG concentrations (0, 1, 4, 6, 10, 12, 14, and 16 $\mu\text{g/ml}$) for 24 h. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich). The optical density of each well was measured using a microplate reader at 550 nm. The experiments were performed in three independent experiments.

α -MG in pluronic lecithin organogel

The aqueous phase was prepared by mixing pluronic F-127 (30% w/v) (Sigma-Aldrich) and potassium sorbate (0.2%) (Sigma-Aldrich) under magnetic stirring at 4°C. The oil phase containing 4 ml isopropyl palmitate (Sigma-Aldrich), lecithin (2% w/v) (DUKSAN Science, Seoul, Korea), and sorbic acid (0.2%) (Sigma-Aldrich) was homogenized under magnetic stirring at room temperature. α -MG (25 mg/ml) was added to the oil phase. To prepare PLO, the aqueous phase, oil phase, and polyethylene glycol 600 (Sigma-Aldrich) were mixed at a ratio of 3.5:1:0.5 (v/v), respectively. All ingredients were homogenized using a luer lock syringe.

Skin infection model

Female BALB/c mice (6 weeks old) were purchased from Hyochang Science (Daegu, Korea). Mice were housed in a flow chamber maintained at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and relative humidity of $55\% \pm 5\%$. The tape-stripping infection model was used for skin infection of *S. pseudintermedius* in mice (Kugelberg et al., 2005). Two sets of four mice in each group were used in this study: one set of mice was used for qPCR and histological analyses, and the other set was used for CFU counting. *S. pseudintermedius* ATCC 49051 was cultured at 37°C for 18 h and diluted 1:100 in trypticase soy broth (Difco). Bacteria were grown to reach an OD_{600} of 0.5 and washed with PBS. After sterilizing the ears using 70% ethanol, the dorsal surface of both ears was stripped 30 times using the autoclave tape to disrupt the epidermal barrier. The bacterial suspension (1.0×10^9 CFU/20 μl PBS) was inoculated on the stripped skin of both ears. For treatment group, α -MG (7.5 μg in 30 μl PLO) was applied four times with 6 h intervals to the infection sites 4 h after bacterial inoculation. Uninfected control was normal mice without the skin stripping, bacterial infection, or any treatment. Infected control mice (control group) were inoculated bacterial suspension on the stripped skin and then treated with DMSO in PLO (0.3 μl DMSO in 30 μl PLO) four times as the same intervals of treatment group. Animal experiments were performed in accordance with the regulations established by the Institutional Animal Care and Use Committee of Kyungpook National University.

Histological analysis

To evaluate histological changes in the ears, mice were euthanized 6 h after the final treatment. The dissected right ears were fixed with 10% paraformaldehyde and embedded in a paraffin block. The paraffin-embedded ears were serially sectioned with 3- μm -thick slices and stained with hematoxylin and eosin (H&E).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (San Diego, CA, USA). One-way analysis of variance with Dunnett's *post-hoc* analysis and Student's *t*-tests were performed to compare the control and experimental groups. Differences of $p < 0.05$ were considered statistically significant.

Results

Antimicrobial activity of α -MG against *Staphylococcus* species and G (-) bacteria from companion animals *in vitro*

To assess the antimicrobial activity of α -MG against G (+) and G (-) bacterial species commonly isolated from skin diseases of companion animals, the MICs of α -MG against eight staphylococcal reference strains and two G (-) reference strains were determined

using agar dilution and broth microdilution methods. The MICs of α -MG against staphylococcal strains ranged from 1 to 2 and 1 to 4 $\mu\text{g}/\text{ml}$ in broth microdilution and agar dilution methods, respectively (Table 1). Five *Staphylococcus* species, including *S. aureus*, *S. felis*, *S. intermedius*, *S. pseudintermedius*, and *S. saprophyticus*, showed two-fold difference in MICs of α -MG between the broth microdilution and agar dilution methods, whereas three species, including *S. carprae*, *S. epidermidis*, and *S. schleiferi*, showed the same MIC values between the two methods. The MBCs of α -MG against *Staphylococcus* species were ranged from 1 to 8 $\mu\text{g}/\text{ml}$ (Table 1). However, the MICs and MBCs of α -MG against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were >64 $\mu\text{g}/\text{ml}$. Next, the antimicrobial activity of α -MG against clinical isolates of *Staphylococcus* species and G (-) bacterial species derived from skin diseases of companion dogs and cats was determined using the agar dilution method. The MICs of α -MG against all clinical isolates of *Staphylococcus* species ranged from 1 to 16 $\mu\text{g}/\text{ml}$, whereas those against G (-) bacterial isolates were >64 $\mu\text{g}/\text{ml}$ (Table 2). The MIC_{50} value of α -MG was the lowest in *S. schleiferi* isolates (4 $\mu\text{g}/\text{ml}$), whereas the MIC_{90} of α -MG was the highest in *S. aureus* isolates (16 $\mu\text{g}/\text{ml}$). Time-killing analysis was performed to understand the nature of the antimicrobial activity of α -MG against *S. felis*, *S. pseudintermedius* and *S. schleiferi*. The growth of the three staphylococcal strains was completely inhibited at the MIC of α -MG for 18 h (Figure 1). These results suggest that α -MG exhibits a strong antimicrobial activity against *Staphylococcus* species from companion animals *in vitro*, but not against G (-) bacteria.

Morphological changes of *Staphylococcus* species treated with α -MG

To determine whether α -MG induced the morphological changes of bacteria, three staphylococcal strains were treated with different α -MG concentrations ($1/4\times$ to $4\times$ MIC) for 2 h, and bacterial morphology was assessed using FE-SEM. No morphological change was observed in bacteria treated with $1/4\times$ MIC of α -MG compared to bacteria treated with DMSO, a solvent of α -MG (Figure 2). However, morphological changes were observed in *S. felis* and *S. pseudintermedius* treated with $\geq 1/2\times$ MICs of α -MG and *S. schleiferi* treated with $\geq 1\times$ MICs of α -MG. Control bacteria showed an intact and smooth surface and a spherical shape, whereas staphylococcal strains treated with $\geq 1\times$ MICs of α -MG showed significant morphological changes such as deep surface craters, cell disruption, and cellular debris.

Specific binding of α -MG to MAP domain-containing protein in the cytoplasmic membrane of *S. pseudintermedius*

To identify bacterial molecules interacting with α -MG, α -MG was labeled with biotin molecules at the hydroxyl group (-OH) of C-3 and/or C-6. The antimicrobial activity of biotinylated α -MG was first determined against *S. felis* ATCC 49168, *S. pseudintermedius* ATCC 49051, and *S. schleiferi* ATCC 43808.

TABLE 2 *In vitro* antimicrobial activity of α -MG against clinical isolates of *Staphylococcus* species and G (-) bacteria from skin lesions of companion dogs and cats.

Bacterial isolates (No.)	No of isolates with the following MIC (μ g/ml)							MIC range (μ g/ml)	MIC ₅₀ (μ g/ml)	MIC ₉₀ (μ g/ml)
	1	2	4	8	16	32	>64			
<i>S. aureus</i> (19)			1	12	6			4-16	8	16
<i>S. epidermidis</i> (27)	1		5	20	1			1-16	8	8
<i>S. felis</i> (51)		1	9	41				2-8	8	8
<i>S. pseudintermedius</i> (80)	1		22	57				1-8	8	8
<i>S. schleiferi</i> (64)	1	3	48	12				1-8	4	8
<i>E. coli</i> (20)							20	>64	>64	>64
<i>P. aeruginosa</i> (16)							16	>64	>64	>64

*MIC, minimum inhibitory concentration; MIC₅₀, MIC of 50% of the isolates; MIC₉₀, MIC of 90% of the isolates.

The MICs of α -MG labeled with one biotin molecule at either C-3 or C-6 increased from 1 to 32 μ g/ml in the three staphylococcal strains using the broth microdilution method. Moreover, the MICs of α -MG labeled with two biotin molecules at both sites increased to 32 μ g/ml in *S. felis* ATCC 49168, 64 μ g/ml in *S. schleiferi* ATCC 43808, and 128 μ g/ml in *S. pseudintermedius* ATCC 49051. These results suggest that hydroxyl groups at C-3 and C-6 are essential for the antimicrobial activity of α -MG against *Staphylococcus* species. Next, a protein pull-down assay was performed using biotinylated α -MG. Streptavidin gels were used in this assay due to the high affinity of biotin to streptavidin. Whole cell lysates of *S. pseudintermedius* ATCC 49051 were incubated with α -MG labeled with one biotin molecule bound to streptavidin gels. Bacterial proteins with ~27 kDa were identified to interact with

biotinylated α -MG in SDS-PAGE gels (Figure 3A). However, no protein band was observed when bacterial lysates were incubated with α -MG labeled with two biotin molecules at C-3 and C-6 (Figure S3). Four bacterial proteins were identified (Table 3). The MAP domain-containing protein was the most abundant of the four proteins and was predicted to be located in the cytoplasmic membrane using the PSORTb program. To determine the exact localization of MAP domain-containing protein in bacteria, whole cell lysates, cell wall-associated proteins, and MVs were purified from *S. pseudintermedius* ATCC 49051, and western blot analysis was performed using the mouse anti-MAP domain-containing protein polyclonal serum. This revealed that the MAP domain-containing protein was located in the cell wall, but this protein was merely secreted by MVs (Figure 3B). These results suggest that α -

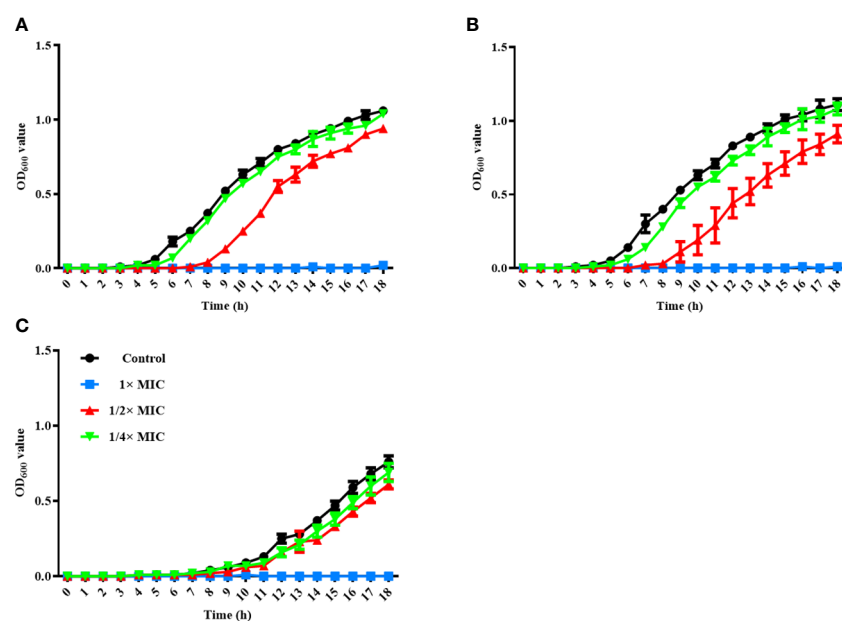


FIGURE 1

Time-kill kinetics assay for three *Staphylococcus* species. *S. felis* ATCC 49168 (A), *S. pseudintermedius* ATCC 49051 (B), and *S. schleiferi* ATCC 43808 (C) were grown in Mueller-Hinton broth with different α -MG concentrations (1/4x, 1/2x, and 1x MICs) for 18 h. OD₆₀₀ was measured at the indicated time points. The data are presented as the mean $\pm$ SD of three independent experiments. The MIC values of α -MG against three *Staphylococcus* strains were 1 μ g/ml using the broth microdilution method.

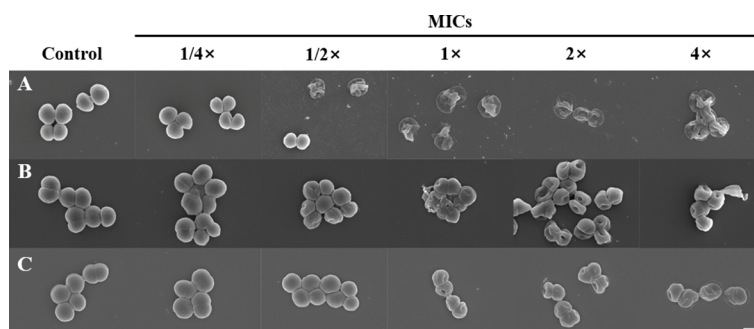


FIGURE 2

Morphological changes of bacteria treated with α -MG. *S. felis* ATCC 49168 (A), *S. pseudintermedius* ATCC 49051 (B), and *S. schleiferi* ATCC 43808 (C) were treated with 1/4x, 1/2x, 1x, 2x, and 4x MICs of α -MG for 2 h, and bacterial morphology was analyzed using FE-SEM. The control bacteria were treated with 0.16% DMSO. The MIC values α -MG against three *Staphylococcus* strains were 1 μ g/ml using the broth microdilution method. Magnification, x20,000. Scale bar, 1 μ m.

MG interacts with MAP domain-containing protein located in the cytoplasmic membrane of *S. pseudintermedius* via hydroxyl groups at C-3 and C-6.

Antimicrobial activity of α -MG by the interaction with MAP domain-containing protein

To determine whether the antimicrobial activity of α -MG was mediated by the interaction with the MAP domain-containing protein, *S. pseudintermedius* ATCC 49051 was pretreated with mouse anti-MAP domain-containing protein polyclonal serum for 20 min and incubated with different α -MG concentrations (1x, 2x, and 4x MICs) for 20 min. As a control, bacteria were pretreated with mouse pre-immune serum and treated with α -MG. No significant difference in the CFU of bacteria treated with 1x and

2x MICs of α -MG was observed between the pretreatment of pre-immune serum and anti-MAP domain-containing protein polyclonal serum (Figure 4). α -MG with 4x MIC completely killed bacteria pretreated with pre-immune serum within 20 min, but the same α -MG concentration reduced the CFU <10-fold in bacteria pretreated with anti-MAP domain-containing protein polyclonal serum. These results suggest that the antimicrobial activity of α -MG against *S. pseudintermedius* is directly mediated by the interaction with MAP domain-containing proteins.

Gene expression of *S. pseudintermedius* ATCC 49051 treated with sub-MIC of α -MG

To investigate the gene expression of *S. pseudintermedius* exposed to sub-MIC of α -MG, *S. pseudintermedius* ATCC 49051

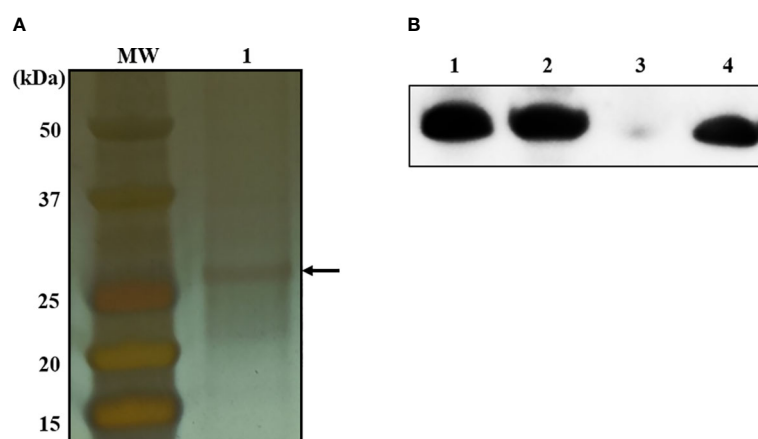


FIGURE 3

Bacterial proteins interacting with α -MG labeled with one biotin molecule at C-3 or C-6 and subcellular localization of MAP domain-containing protein. (A) Whole cell lysates of *S. pseudintermedius* ATCC 49051 were added to streptavidin gels bound to α -MG labeled with one biotin molecule. Bacterial proteins bound to biotinylated α -MG were observed in silver-stained SDS-PAGE gel. Arrow indicates bacterial proteins bound to biotinylated α -MG. MW, molecular weight size marker; 1, bacterial proteins bound to biotinylated α -MG. (B) Subcellular localization of MAP domain-containing protein using western blot analysis. Bacterial proteins of *S. pseudintermedius* ATCC 49051 were separated by 10% SDS-PAGE and western blot was performed using the mouse anti-MAP domain-containing protein polyclonal serum. Lanes 1, whole cell lysates; 2, cell wall-associated proteins; 3, membrane vesicles; 4, recombinant MAP domain-containing proteins.

TABLE 3 Identification of bacterial proteins bound to biotinylated α -MG using LC-MS.

Gene accession No. ^a	Description of the identified protein	MW (Da)	pI	Signal peptide ^b	Subcellular localization ^c	emPAI ^d
WP_105963101.1	helix-turn-helix transcriptional regulator [<i>Staphylococcus</i>]	25,492	4.89	No	Cytoplasm	0.19
WP_014613648.1	MAP domain-containing protein [<i>S. pseudintermedius</i>]	27,424	9.86	Yes	Cytoplasmic membrane	4.36
WP_110159498.1	SAR2788 family putative toxin [<i>S. pseudintermedius</i>]	27,559	9.48	Yes	Extracellular	0.16
WP_014612681.1	response regulator transcription factor [<i>Staphylococcus</i>]	27,322	5.17	No	Cytoplasm	0.18

^aNCBI reference sequence (<https://www.ncbi.nlm.nih.gov/>).
^bPrediction of signal peptide and cleavage sites by SignalP 3.0 (<https://services.healthtech.dtu.dk/service.php?SignalP-5.0>).
^cPrediction of cellular location by PSORTb v3.0 (<https://psort.org/psortb>).
^dExponentially modified protein abundance index.

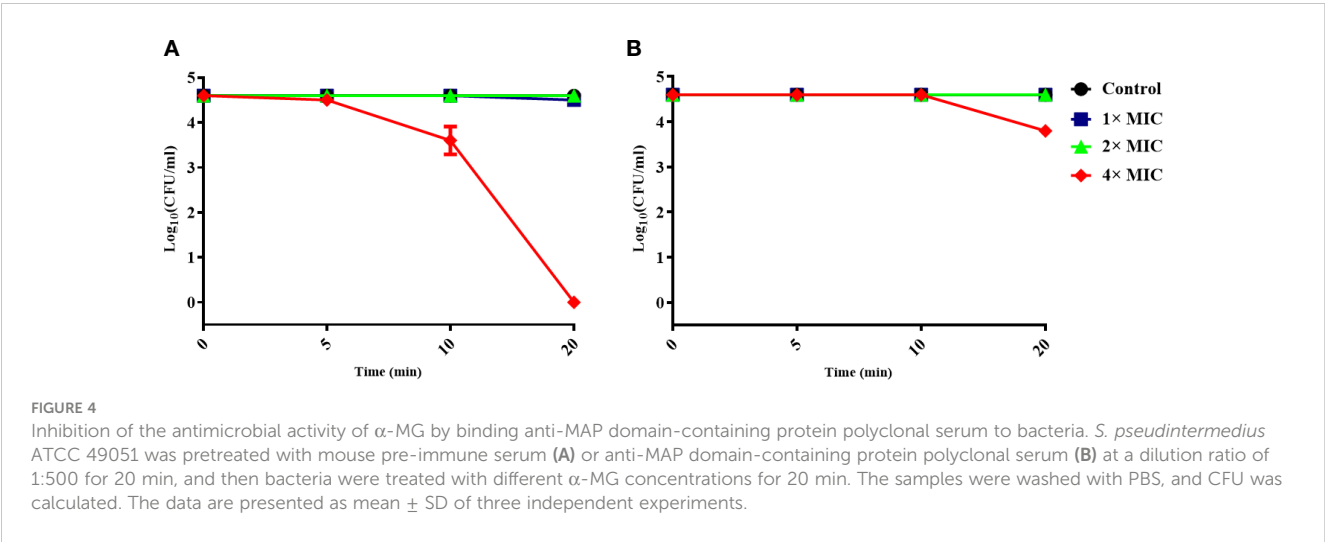
was treated with 0.7× MIC of α -MG for 10 min, and DEGs were analyzed. As a control, bacteria were treated with DMSO. A total of 2,253 genes were identified under control and experimental conditions, corresponding to 91.5% (2,463 genes) of *S. pseudintermedius* genes. Of the 2,253 genes identified, 194 genes, which showed a fold change ≥ 2 or ≤ 0.5 and Wald test $q < 0.05$, were identified as DEGs using DEseq2 analysis (Figure 5A). Upregulated and downregulated genes were 135 and 59, respectively. DEGs of *S. pseudintermedius* ATCC 49051 exposed to sub-MIC of α -MG are listed in Supplementary Table S2. qPCR was performed to verify the expression of DEGs by transcriptome analysis. The four upregulated and downregulated genes showed the same expression pattern observed in the transcriptome analysis (Figure S4). Cytoplasmic proteins were the most abundant (52.6%), followed by cytoplasmic membrane proteins (33.5%) (Figure 5B). Based on KEGG pathways, DEGs involved with metabolic pathways were abundant, followed by biosynthesis of secondary metabolites (Figure 5C). However, many DEGs were found to encode hypothetical proteins. These results suggest that the sublethal concentration of α -MG regulates the expression of many genes, especially in metabolic pathway, in *S. pseudintermedius*.

Host cell toxicity of α -MG *in vitro*

To determine whether α -MG could induce host cell cytotoxicity, CPEKs were treated with different α -MG concentrations (1–16 μ g/ml) for 24 h, and cell viability was determined using the MTT assay. As a control, CPEKs were treated with DMSO. No cytotoxicity was observed at ≤ 12 μ g/ml α -MG, but the cytotoxicity was observed at ≥ 14 μ g/ml α -MG (Figure 6).

Antimicrobial and anti-inflammatory activities of α -MG in skin lesions caused by *S. pseudintermedius* *in vivo*

To determine the therapeutic potentials of α -MG in bacterial skin infections *in vivo*, *S. pseudintermedius* ATCC 49051 was inoculated on the dorsal side of ears in mice, and α -MG in PLO was applied four times at 6 h intervals (Figure 7A). PLO was used for the topical drug delivery vehicle of α -MG. CFU, histological changes, and expression of cytokine genes in skin lesions were



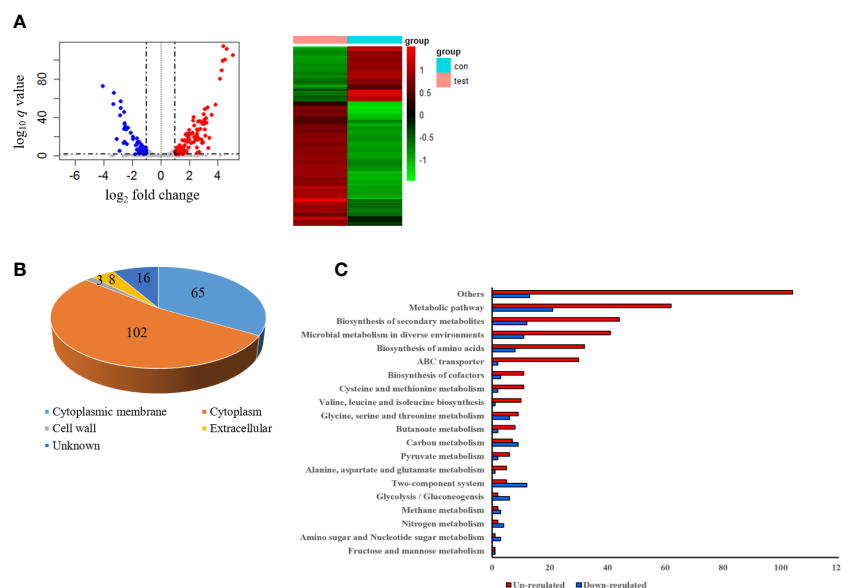


FIGURE 5

Differentially expressed genes (DEGs) in *S. pseudintermedius* ATCC 49051 exposed to sublethal concentration of α -MG. Bacteria were treated with 0.7 $\times$ MIC of α -MG for 10 min, and total RNA was isolated. As a control, bacteria were treated with DMSO. (A) Volcano plot and DEGs. (B) Prediction of subcellular localization of proteins encoded by DEGs. (C) Upregulated and downregulated genes classified by the KEGG pathway.

assessed 6 h after the final treatment. α -MG in PLO reduced the bacterial number by >99% compared to control mice treated with DMSO in PLO (Figure 7B). Infection of *S. pseudintermedius* ATCC 49051 induced the loss of the epidermal barrier and infiltration of cells in skin lesions of the infected control group (Figure 7C). α -MG in PLO reduced the number of infiltrating cells and partially restored the epidermal structure in skin lesions. To determine the anti-inflammatory activity of α -MG in PLO, qPCR was performed to measure the expression of pro-inflammatory (IL-1 β and TNF- α), Th1 (IFN- γ), Th2 (IL-13), and Th17 (IL-17A) cytokine genes. *S. pseudintermedius* infection (control group) significantly induced the expression of all tested cytokine genes, but α -MG in PLO

significantly inhibited the expression of all tested cytokine genes like uninfected control group (Figure 7D). These results suggest that α -MG in PLO exhibits antimicrobial and anti-inflammatory activities in skin lesions caused by *S. pseudintermedius* *in vivo*.

Discussion

The antimicrobial activity of α -MG against G (+) bacterial pathogens, including *S. aureus*, *S. epidermidis*, *Enterococcus faecalis*, and *Streptococcus gordonii* from human clinical specimens, has already been demonstrated (Sakagami et al., 2005; Sivaranjani et al., 2017; Felix et al., 2022; Leelapornpisid, 2022). However, the antimicrobial activity of α -MG against *Staphylococcus* species originating from companion animals has not been characterized. The MICs of α -MG against reference strains of *Staphylococcus* species and G (-) bacterial species were first determined using the broth microdilution and agar dilution methods. There was no or 2-fold difference in MICs of α -MG between the two methods. Time-kill kinetics showed the different growth inhibition pattern of *S. schleiferi* treated 1/2 MIC of α -MG compared to that of *S. pseudintermedius* and *S. felis* with 1/2 MIC of α -MG. This is partly due to slow growth of *S. schleiferi* *in vitro* culture. The agar dilution method was applied to determine the MICs of α -MG against 241 isolates of five different *Staphylococcus* species from skin diseases of companion dogs and cats. The distribution of MICs of α -MG against clinical isolates of all tested *Staphylococcus* species represents a strong antimicrobial activity of α -MG *in vitro*. However, α -MG showed high MICs (>64 μ g/ml) against clinical isolates of *E. coli* and *P. aeruginosa* from companion animals. These results suggest that α -MG exhibits antimicrobial activity against *Staphylococcus* species from companion animals as well as G (+)

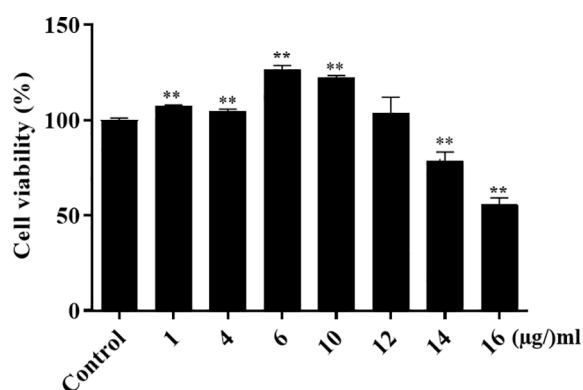


FIGURE 6

Viability of canine progenitor epidermal keratinocytes treated with α -MG. Cells were treated with different α -MG concentrations (1–16 μ g/ml) for 24 h, and MTT assays were performed. Control cells were treated with 0.13% DMSO. The data are presented as mean $\pm$ SD of three independent experiments. ** p < 0.01 compared to control cells.

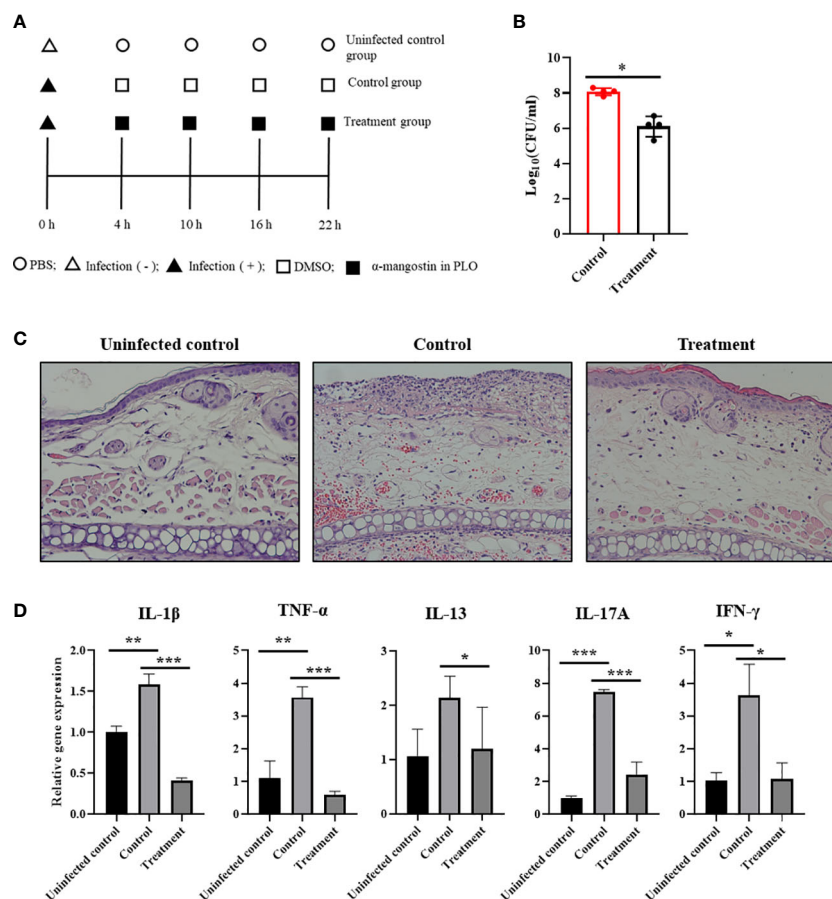


FIGURE 7

Antimicrobial and anti-inflammatory activities of α -MG in skin lesions induced by *S. pseudintermedius* in a murine model. (A) Experimental schedule for the application of α -MG. Mice were infected with 1.0×10^9 CFUs/20 μ l *S. pseudintermedius* ATCC 49051 and treated with α -MG in PLO. Infected control mice (control) were treated with DMSO in PLO. (B) Antimicrobial activity of α -MG in the tape-stripping infection model. The data are presented as mean of CFUs $\pm$ SD of four mice. * $p < 0.05$ compared to the control group. (C) Histopathological assessment of mouse ear. The ear section was stained with H&E. Magnification, $\times 200$. (D) Expression of cytokine genes using qPCR. The data are presented as mean $\pm$ SD of four mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to uninfected or infected control group.

bacterial pathogens from humans (Sakagami et al., 2005; Sivarajani et al., 2017; Felix et al., 2022; Leelapornpisid, 2022).

To understand the action mechanisms of α -MG against *Staphylococcus* species, FE-SEM was used to determine the morphological changes of bacteria treated with α -MG. Interestingly, ultrastructural alterations of bacteria treated with α -MG such as surface craters, surface indentation, cell disruption, and cellular debris were comparable to the results from *S. aureus* treated with antimicrobial peptides (Wu and Hancock, 1999; Tan et al., 2019; Deshmukh et al., 2021) or membrane acting antimicrobials such as daptomycin (Silverman et al., 2003), suggesting that α -MG targets the cytoplasmic membrane of *Staphylococcus* species. α -MG causes considerable damage to membrane integrity, whereas membrane acting antimicrobials produce ion channels or transmembrane pores (Wu and Hancock, 1999; Silverman et al., 2003; Cotroneo et al., 2008). Koh et al. (2013) also demonstrated that α -MG directly interacts with the cytoplasmic membrane of *S. aureus* via non-electrostatic interactions, causing bacterial lysis. However, the specific targets of α -MG in the cytoplasmic membrane have not been determined. To identify the specific interacting molecules of α -MG in the cytoplasmic

membrane of *S. pseudintermedius*, hydroxyl groups of α -MG at C-3 and C-6 were substituted with biotin molecules. The hydroxyl groups of α -MG at C-3 and C-6 were selected because acetylation at C-3 and C-6 significantly changed the pharmacological activities of α -MG such as antioxidant and anticancer properties in human cells (Tran et al., 2021). Methylation at C-3 and C-6 in α -MG and γ -mangostin also reduced antimicrobial activity against G (+) bacteria (Tran et al., 2021). However, α -MG maintained antimicrobial activity when an appropriate cationic moiety or aliphatic amine was attached to the hydrophobic core and lipophilic chains instead of hydroxyl groups (Zou et al., 2013; Koh et al., 2015). The antimicrobial activity of biotinylated α -MG was first tested against three *Staphylococcus* species. Biotinylation α -MG at C-3 and C-6 showed lower antimicrobial activity against *Staphylococcus* species than α -MG. The MICs of α -MG with two biotin molecules were higher than those of α -MG with one biotin. Moreover, α -MG with one biotin molecule interacted with bacterial proteins, whereas no bacterial proteins were found to interact with α -MG with two biotin molecules. These results suggest that α -MG interacts with bacterial proteins via hydroxyl groups at C-3 and C-6.

In this study, MAP domain-containing protein was identified to interact with α -MG. MAP shows that six repeated domains of 110 residues containing a subdomain of 31 residues are highly homologous to the N-terminal β -chain of many MHC class II molecules (Jonsson et al., 1995; Kreikemeyer et al., 2002; Hagggar et al., 2003). Mice infected with the *map*-negative mutant strain exhibit a reduced kidney abscess formation compared to mice infected with the wild-type *S. aureus* strain (Lee et al., 2002). MAP may attenuate cellular immunity and potentiate *S. aureus* survival by modulating host immunity. However, the MAP domain-containing protein identified in *Staphylococcus* species has not been characterized yet. The MAP domain-containing protein was predicted to carry a signal peptide (1-30 amino acids) and two MAP domain regions (37-147 and 148-247 amino acids) using the AlphaFold program (Figure S5). The sequence homology of amino acids was searched to determine whether MAP domain-containing protein was identified only in G (+) bacteria because α -MG exhibited antimicrobial activity against only G (+) bacteria, but not G (-) bacteria. The amino acid sequence of the MAP domain-containing protein identified in *S. pseudintermedius* ATCC 49051 was similar to 61%, 33%, 33%, and 30% in *Staphylococcus*, *Bacillus*, *Streptomyces*, and *Clostridium* species, respectively, using the UniProt BLAST program (Figure S6). However, this protein did not show any similarity to proteins in G (-) bacteria. These results may partly explain the antimicrobial activity of α -MG against G (+) bacteria, but not G (-) bacteria. Blocking the MAP domain-containing protein in the cytoplasmic membrane of *S. pseudintermedius* by anti-MAP domain-containing protein polyclonal serum significantly reduced the antimicrobial activity of α -MG. For the first time, this study demonstrated that α -MG specifically interacts with MAP domain-containing protein in the cytoplasmic membrane of *S. pseudintermedius*, resulting in cell lysis.

The subinhibitory concentrations of α -MG change the expression of many genes in *S. epidermidis* (Sivaranjani et al., 2019). Transcriptomic and proteomic analyses of *S. epidermidis* treated with sub-MIC (0.7 $\times$) of α -MG show downregulation of genes conferring cytoplasmic membrane integrity, cell division, teichoic acid biosynthesis, fatty acid biosynthesis, biofilm formation, and DNA replication and repair machinery, whereas sub-MIC of α -MG upregulates the genes associated with oxidative and cellular stress responses. In this study, the sub-MIC (0.7 $\times$) of α -MG modulated the expression of 194 genes in *S. pseudintermedius*, especially genes associated with metabolic pathways. α -MG upregulated genes encoding fatty acid biosynthesis, fatty acid degradation, fatty acid metabolism, pyrimidine (*carB*, *dcd*, *pyrD*, *pyrE*, *pyrF*, and *pyrK*), nucleotide metabolism (*dcd*), and CoA biosynthesis (*ilvB*, *ilvD*, *ilvE*, and *ilvN*), essential for bacterial survival. α -MG downregulated genes encoding pentose phosphate (fructose bisphosphate aldolase, *pfkA*), peptidoglycan biosynthesis (*sgtB* and *spsN*), teichoic acid biosynthesis (*dltX*), and citrate cycle (2-oxoacid: acceptor oxidoreductase subunit α). In addition, α -MG upregulated genes associated with siderophores (*FhuD*, *sbnA*, *sbnB*, *sbnC*, *sbnE*, and *sbnF*), but downregulated virulence determinants, including exotoxin (SpEX), β -channel forming cytolysin (leukocidin), leukocidin/hemolysin toxin family protein, and

coagulase (Cheng et al., 2010; Abouelkhair et al., 2018 and Abouelkhair et al., 2019). These results suggest that sublethal concentrations of α -MG can modulate genes associated with the metabolic pathway, cell wall biosynthesis, and virulence factors in *S. pseudintermedius*, although the cytoplasmic membrane is a primary target of α -MG. It should be determined whether the transcriptional regulation of α -MG is a direct effect induced by the interaction with regulatory molecules in the cytoplasm or an indirect effect induced by the interaction with bacterial molecules in the cytoplasmic membrane.

The tape-stripping infection model was used to determine the therapeutic potential of α -MG *in vivo*, because *Staphylococcus* species was commonly associated with superficial and surface pyoderma in companion animals (Empinotti et al., 2012). By inoculation of *S. pseudintermedius*, high CFUs were maintained and infiltrating cells were remarkably observed in skin lesions. A relatively low dose of α -MG (7.5 μ g in PLO) was applied in skin lesions because host cell cytotoxicity was induced in CPEKs treated with ≥ 14 μ g/ml α -MG *in vitro*, and MICs of α -MG against all clinical isolates of *S. pseudintermedius* were ≤ 8 μ g/ml. α -MG induced cellular proliferation of CPEKs treated with 1-10 μ g/ml α -MG. α -MG has an antitumor activity against various types of cancer cells, but α -MG induces proliferation of non-tumorigenic human keratinocytes (HaCaT cells) after ultraviolet B (UVB) radiation (Im et al., 2017). α -MG may modulate innate immunity by the proliferation of keratinocytes induced by *S. pseudintermedius* infection like UVB radiation. α -MG in PLO significantly reduced bacterial number in skin lesions. In addition, α -MG in PLO significantly decreased the expression of cytokine genes associated with inflammation and adaptive immunity. Partial restoration of the epidermal structure and disappearance of infiltrated cells were observed in skin lesions treated with α -MG in PLO. These results suggest that α -MG in PLO exerts a synergistic effect on *S. pseudintermedius*-induced skin lesions via antimicrobial and anti-inflammatory activities in a murine model. Xanthones modulate inflammatory responses and the expression of inflammatory cytokines (Aye et al., 2020; Gunter et al., 2020). Oral administration of mangosteen rind extract inhibits the expression of pro-inflammatory cytokine and atopic dermatitis-associated genes and restores the epidermal barrier in an atopic dermatitis murine model (Higuchi et al., 2013). In addition, xanthones, including α -MG, exhibit anti-acne activity induced by *Cutibacterium acnes* (Chomnawang et al., 2007; Xu et al., 2018). However, complete clearance of bacteria was not observed in skin lesions, although α -MG concentrations exceeded the MIC level against *S. pseudintermedius* ATCC 49051. The lower antimicrobial activity of α -MG against *S. pseudintermedius* *in vivo* than its antimicrobial activity *in vitro* may be due to insolubility or low binding of α -MG to bacterial surface in skin lesions.

In conclusion, α -MG exhibited antimicrobial activity against clinical isolates of *Staphylococcus* species from companion animals *in vitro*. α -MG specifically interacted with MAP domain-containing protein in the cytoplasmic membrane of *S. pseudintermedius* via hydroxyl groups at C-3 and C-6. In addition, sublethal concentrations of α -MG inhibited the expression of genes conferring virulence factors in *S. pseudintermedius*. α -MG in PLO

was an effective treatment modality in skin lesions caused by *S. pseudintermedius* via antimicrobial and anti-inflammatory activities. The development of new α -MG therapeutics is a novel approach to treating *Staphylococcus*-induced skin infections in companion animals, reducing the use of conventional antimicrobial agents.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA963078.

Ethics statement

Animal experiments were performed in accordance with the regulations established by the Institutional Animal Care and Use Committee of Kyungpook National University.

Author contributions

SYP, JHL, and JCL designed the experiments. SYP, SYK, NK, and SYK carried out the experiments. JCL directed research and provided insightful discussions. All authors contributed to the analysis of the data, revised the manuscript, and approved its final version.

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Polymyxin B complexation enhances the antimicrobial potential of graphene oxide

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Introduction: The antibacterial activity of graphene oxide (GO) has been widely explored and tested against various pathogenic bacterial strains. Although antimicrobial activity of GO against planktonic bacterial cells was demonstrated, its bacteriostatic and bactericidal effect alone is not sufficient to damage sedentary and well protected bacterial cells inside biofilms. Thus, to be utilized as an effective antibacterial agent, it is necessary to improve the antibacterial activity of GO either by integration with other nanomaterials or by attachment of antimicrobial agents. In this study, antimicrobial peptide polymyxin B (PMB) was adsorbed onto the surface of pristine GO and GO functionalized with triethylene glycol.

Methods: The antibacterial effects of the resulting materials were examined by evaluating minimum inhibitory concentration, minimum bactericidal concentration, time kill assay, live/dead viability staining and scanning electron microscopy.

Results and discussion: PMB adsorption significantly enhanced the bacteriostatic and bactericidal activity of GO against both planktonic cells and bacterial cells in biofilms. Furthermore, the coatings of PMB-adsorbed GO applied to catheter tubes strongly mitigated biofilm formation, by preventing bacterial adhesion and killing the bacterial cells that managed to attach. The presented results suggest that antibacterial peptide absorption can significantly enhance the antibacterial activity of GO and the resulting material can be effectively used not only against planktonic bacteria but also against infectious biofilms.

KEYWORDS

carbon materials, antibiotics, bacteria, adhesion, biofilm, antimicrobial peptide

1 Introduction

Bacteria grow either as solitary cells in planktonic form or as multicellular structures known as biofilms. In comparison to planktonic cells, life in a biofilm increases bacterial survival in adverse conditions and promotes pathogenicity as protects bacteria from the immune system (Donlan and Costerton, 2002; Helgadóttir et al., 2017; Sharma et al., 2019). In a world where antibiotic-resistant harmful bacteria are proliferating at an alarming rate, finding solutions to counter the infections has become a major public health concern (Aslam et al., 2018). Unfortunately, very little has been achieved in combating this problem through the discovery of new antibiotics (Miethke et al., 2021).

Graphene oxide (GO) has been reported for its antimicrobial activity against a range of Gram-positive and Gram-negative bacterial strains (Liu et al., 2011; Chen et al., 2014; Romero et al., 2020). The antimicrobial activity of GO is mainly due to cell wrapping/trapping, insertion, damage of cell membrane, and generation of oxidative stress (Zou et al., 2017; Pulingam et al., 2019; Chen et al., 2021; Ravikumar et al., 2022). Our recent study demonstrated the correlation of time-dependent bacteriostatic and bactericidal effects of GO with changes in the cell membrane and expression of stress-associated proteins (Ravikumar et al., 2022). However, the antimicrobial intensity of pristine GO alone is not sufficient to kill bacterial cells on a large scale and inside bacterial communities such as biofilms (Pandit et al., 2021). This is limiting the application of GO in clinical settings, for the treatment of infectious lesions or prevention of bacterial biofilm formation on biomedical devices (Fallatah et al., 2019; Di Giulio et al., 2020). The antimicrobial performance of GO is heavily affected by the size, shape, thickness, surface charge, purity, and as well as dispersion status of GO nanosheets (Liu et al., 2012; Perreault et al., 2015; Barbolina et al., 2016; Anand et al., 2019). Some of these properties can be improved, and there exists considerable interest in combining GO with other nanomaterials or antimicrobial compounds to enhance its antimicrobial activity (Bugli et al., 2018; Singh et al., 2019; Han et al., 2020; Shamsi et al., 2022).

The cyclic cationic peptide polymyxin B (PMB), which is specific for Gram-negative bacteria, has been used as a “last resort” antimicrobial (Goode et al., 2021; Maynard et al., 2021). The antimicrobial activity of PMB is associated with cell membrane damage, inhibition of respiration, generation of reactive oxygen species, and inhibition of cell division (Mortensen et al., 2009; Trimble et al., 2016; Brennan-Krohn et al., 2018; Ayoub Moubareck, 2020). Specifically, in Gram-negative bacteria, the cationic peptide region of PMB binds electrostatically to the negatively charged lipopolysaccharide of the outer membrane (Fernández et al., 2013; Ayoub Moubareck, 2020; Manioglu et al., 2022). The hydrophobic fatty acid chains then interact with lipid A of the lipopolysaccharides, resulting in loss of membrane stability, facilitating the uptake of PMB into the outer membrane (Landman et al., 2008; Trimble et al., 2016). This phenomenon ultimately results in the weakening of the outer membrane and alters cell permeability. The disruption in cell membrane permeability results in the release of periplasmic proteins, damaging the cells (Yu et al., 2015; MacNair et al., 2018). In

addition to affecting the membrane, it has been demonstrated that PMB exposure can generate reactive oxygen species (ROS) including superoxide, hydrogen peroxide, and hydroxyl radicals (Kohanski et al., 2007; Ayoub Moubareck, 2020). ROS causes oxidative damage of cellular components such as lipids, nucleic acids, and proteins, leading to cell death. We hypothesize that PMB combining with GO could be able to enhance the antimicrobial potential of graphene oxide. PMB was chosen due to its activity against Gram-negative strains that are resistant to other antibiotics, and due to its mode of action.

In this study, the natural PMB produced by *Bacillus polymyxa* (sulfate salt containing PMB B1 and B2. PMB B1 and PMB B2 contain an ethyl and a methyl group in the lipid chain, respectively) (Meng et al., 2016) was physisorbed onto the surface of pristine or functionalized GO (Guo et al., 2022). As GO colloidal stability decreased after PMB complexation, the surface of GO was modified by exploiting the epoxide ring opening to graft the amino triethylene glycol (TEG) chain. We describe the synthesis and characterization of GO and TEG-functionalized GO in combination with PMB, and we assess the capacity of the resulting materials to eradicate bacterial biofilms. Adsorbing PMB to the surface of GO drastically improved the bactericidal properties of GO, including its capacity for the destruction of biofilms made by Gram-negative pathogens *Pseudomonas aeruginosa* and *Escherichia coli*.

2 Materials and methods

2.1 Materials

GO was synthesized following a modified Hummers’ method (Morimoto et al., 2016) and obtained as an aqueous dispersion with a concentration of 3 mg/mL. The solvents were obtained from commercial suppliers and used without purification. Water was purified using a Millipore MilliQ® filter system equipped with the free endotoxin Polysieur Biopak. When stated, the suspensions were sonicated in a water bath Elmasonic P sonicator with settings at 20 W and 40 kHz. MWCO 12,000–14,000 Da dialysis membranes were purchased from Spectrum Laboratories, Inc. Polymyxin B sulfate salt (mixture of polymyxin B1 and B2) and 2,2’-(ethylenedioxy)bis(ethylamine) were purchased from Sigma Aldrich. Depending on the types of containers employed, either 50 mL conical tubes or 1.5 mL microtubes, two centrifuges (Eppendorf Centrifuge 5804R or Eppendorf Centrifuge 5415R) were used.

2.2 Functionalization of graphene oxide and antibiotic adsorption

2.2.1 Synthesis of GO-TEG

GO flakes were dispersed at a concentration of 1.43 mg/mL in endotoxin-free MilliQ® water for the functionalization with 2,2’-(ethylenedioxy)bis(ethylamine) (TEG). The GO dispersion (7 mL) was stirred with Ultra-turrax t10 at low speed to reduce the agglomeration of the flakes during the functionalization process

(Reina et al., 2019). TEG (3mg) in endotoxin-free MilliQ® water (3 mL) was added dropwise using a pipette to reach a final concentration of GO of 1 mg/mL. After the TEG addition, the reaction was further magnetically stirred for 24 h. The unreacted TEG was eliminated by dialysis using endotoxin-free MilliQ® water for 2 days. X-ray photoelectron spectroscopy (XPS), Thermogravimetric analysis (TGA), and Kaiser test analyses were used to determine the loading of TEG (Kaiser et al., 1970). Although this test is commonly employed in peptide synthesis, it has been adapted for use in carbon compounds (Ménard-Moyon et al., 2011; Jarre et al., 2014).

2.2.2 Complexation with polymyxin B

Ultra-turrax t10 was used to stir GO and GO-TEG suspensions in Milli-Q® at low speed (power 3) followed by the addition of a mixture of PMB B1 and B2 diluted in MilliQ® water solution to reach a mass ratio of GO/PMB at 1:1. To allow the complexes to form, the suspensions were left under stirring at room temperature for 12 h. Then, the dispersions were centrifuged at 5000 rpm and washed with MilliQ® water several times until the supernatant contained no PMB (as analyzed by HPLC). The clean conjugates (GO-TEG-PMB and GO-PMB) were freeze-dried. XPS and TGA analyses were used to determine the loading of PMB.

2.3 Characterization of materials

Morphological analyses were performed using a transmission electron microscope (Hitachi 7500, Hitachi High Technologies Corporation, Tokyo, Japan). The images were analyzed with FIJI software. TGA was performed on a TGA1 (Mettler Toledo) apparatus from 30°C to 900°C with a ramp of 10°C/min under N₂ using a flow rate of 50 mL/min and platinum pans. For GO materials, samples were lyophilized before analysis. XPS analysis was performed on a Thermo Scientific K X-ray photoelectron spectrometer with a basic chamber pressure of 10⁻⁸-10⁻⁹ bar and an Al anode as the X-ray source (1486 eV). The samples were analyzed as powder pressed onto a scotch tape (3MTM EMI Copper Foil Shielding Tape 118). Spot size of 400 µm was used for analysis. The survey spectra were an average of 10 scans with a pass energy of 200 eV and a step size of 1 eV. For each sample, the analysis was repeated three times. A flood gun was turned on during the analysis. We grouped the functional groups of C1s spectra to avoid imprecision due to the proximity of the peak values and the overlapping of some bounds. Therefore, the C1s spectra were deconvoluted in C=O (287.6-289.9 eV) for carbonyl groups, C-O (286.2-287.2 eV) for hydroxyl and epoxide groups, C-N (285.9-286.2 eV) for the amine group and sulfur-containing groups and C-C (284.4-285.3 eV) for sp² and sp³ carbon atoms. For the C-N peak, a range of error was considered since the binding energy of this bond overlaps with the C-O and C-C areas. For data analysis, CasaXPS (2.3.18) software was used. A Shirley background subtraction was applied. A line shape of 70% Gaussian/30% Lorentzian [GL(30)] was selected for all peaks. The FWHM was constrained to be the same for all peaks, apart from the π-π* peak

because it is a broad signal. The N1s spectra were deconvoluted into two main peaks at 399.5 eV (N-R₂) for the amine and 401.5 eV (N⁺-R₃) for the ammonium. Infrared spectra of GO were recorded using an attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) Alpha spectrometer from Bruker, with a diamond crystal as a refractive element, in the range 500-4000 cm⁻¹ at a resolution of 4 cm⁻¹.

2.4 Assessing the stability of GO-TEG-PMB complexation

Two mg of solid GO-TEG-PMB was dispersed into 1 mL of fresh LB medium (sonication bath; Elmasonic P30H, 37 kHz). The solution was split into two microcentrifuge tubes (1.5 mL) and kept in the dark at 37°C for 1 day for one of the tubes and 7 days for the other tube. Each microtube was centrifuged at 12000 rpm for 15 min and the supernatant was analyzed by HPLC. HPLC analyses were performed on a Waters Alliance e2695 separations module instrument equipped with an autosampler and 2998 PDA detector, on a Nucleosil C18 column (150×4.6 mm), using a 20 min linear gradient of 0.1% TFA in water to 31% or 100% of 0.08% TFA in acetonitrile at a flow rate of 1.2 mL·min⁻¹.

2.5 Antimicrobial activity of the functionalized GO-PMB complex

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of tested agents were determined by microdilution method as described previously (Cai et al., 2015). Briefly, overnight grown cultures of *P. aeruginosa* and *E. coli* were appropriately diluted, and 100 µL of diluted bacterial suspensions were mixed with a serial dilution of nanomaterials. The bacterial concentration in the mixtures was 1–2×10⁵ colony-forming units (CFU)/mL, and the concentration of tested agents ranged from 1.56 to 100 µg/mL in a series of twofold dilutions. The samples were incubated at 37°C for 24 h, and optical density at 600 nm (OD₆₀₀) was measured. The MIC was defined as the lowest concentration of tested agent which inhibited bacterial growth. For defining the MBC, 10 µL of mixtures described earlier (bacteria and nanomaterials) were placed on agar plates and incubated at 37°C overnight. MBC values were defined as the lowest concentration of the tested agent which prevented the growth of bacteria on agar plates. The treatment time dependent bactericidal efficiency of GO, GO-TEG, and PMB complexed to the different GO was tested against the planktonic cells of *P. aeruginosa* and *E. coli*. To prepare a bacterial inoculum, single colonies of *P. aeruginosa* and *E. coli* were inoculated to 5 mL of LB medium and incubated overnight at 37°C with continuous agitation. For this assay, 20 µL of inoculum (2–5×10⁷ CFU/mL bacterial cells) from overnight grown bacterial cultures were inoculated into fresh LB medium containing sterile deionized water (control), MIC, and 2 times MIC concentration of tested agents. All samples were incubated at 37°C with continuous agitation for 24 h. A fraction of culture (100 µL) from each sample

was taken to determine bacterial viability at time points of 0, 4, 8, and 24 h. The collected samples were serially diluted in 0.89% of a saline buffer. From the diluted samples, 100 μ L were plated onto LB agar plates, incubated at 37°C for 24 h, and the number of colonies was counted.

2.6 Antibiofilm activity of GO-PMB complexes

Biofilms of *E. coli* and *P. aeruginosa* were formed on 15 mm cover glass. Briefly, overnight grown bacterial culture was diluted with fresh LB to make final inoculum 2×10^6 – 5×10^6 CFU/mL. 300 μ L of bacterial inoculum were loaded to cover glass and incubated at 37°C without any disruption to achieve biofilm formation. After 24 h, the culture medium was replaced with fresh LB medium (control) or MIC and 2×MIC of tested agents, and the samples were further incubated for 24 h. After 24 h of treatment, biofilms were collected in 5 mL of 0.89% of NaCl and homogenized by sonication. The homogenized biofilm suspensions (100 μ L) were serially diluted and plated on LB agar plates and incubated overnight at 37°C. The number of colonies was counted to determine the viability of bacterial cells (CFUs). To visualize the live and dead cells, control biofilms and biofilms treated with test agents were stained for 20 min with a mixture of 6.0 μ M SYTO 9 and 30 μ M propidium iodide from a live/dead BacLight Viability kit L13152 (Thermo Fisher Scientific). Fluorescence microscopic imaging of the biofilms was performed using a Zeiss fluorescence microscope (Axio Imager.Z2m; Carl Zeiss Meditec AG, Jena, Germany). Scanning electron microscopy (SEM) analysis of biofilms was performed as described previously (Pandit et al., 2020). Briefly, biofilms were fixed with 3% of glutaraldehyde for 2 h and dehydrated with graded series of ethanol concentrations (40%, 50%, 60%, 70%, 80%, and 90% for 15 min each) and with absolute ethanol for 20 min. The dehydrated biofilm samples were dried at room temperature overnight and coated with gold before SEM imaging, performed with a Supra 60 VP microscope (Carl Zeiss AG, Jena, Germany).

2.7 Testing GO- PMB complexes as catheter coating

In order to deploy functionalized GO-PMB as a surface coating, a commercially available catheter was purchased, sliced into pieces (12 mm) and sonicated for 2 h with the sterile water containing 100 μ g/mL of GO and GO-TEG, 2×MIC concentration of PMB complexed to GO and GO-TEG (Sonication bath, 100% of amplitude). The coated catheter tubes were dried, and UV sterilized before testing the biofilm formation. The inoculum for biofilm formation was prepared by using overnight grown culture of *P. aeruginosa* and *E. coli* and it was diluted with fresh LB to make the final inoculum 2×10^6 – 5×10^6 CFU/mL. The coated and non-coated catheter tubes were individually placed in culture tube containing 2 mL of inoculum. The culture tubes were incubated at 37°C for 24 h. After 24 h, biofilm on catheter tube was dip washed

using sterile water and fixed with 3% of glutaraldehyde for 2 h. After fixation samples were dehydrated with graded series of ethanol concentrations (40%, 50%, 60%, 70%, 80%, and 90% for 15 min each) and with absolute ethanol for 20 min. The dried samples were sputter coated with gold and analyzed by SEM imaging.

2.8 Statistical analysis

All microbiology data are presented as the mean $\pm$ standard deviation from at least three different biological replicates. Intergroup differences were estimated by one-way analysis of variance (ANOVA), followed by a *post hoc* multiple comparison (Tukey) test to compare the multiple means. Differences between values were considered statistically significant when the *P*-value was < 0.05 .

3 Results

3.1 Synthesis and characterization of functionalized GO

The starting GO was obtained from a modified protocol of Hummers oxidation of graphite, leading to a stable aqueous dispersion at the concentration of 3 mg/mL. According to TEM images, single layer GO had an average size of 1.49 ± 0.9 μ m (Figure S1). The adsorption of PMB on the surface of GO was carried out at room temperature by mixing the two components in aqueous solutions (Figure 1). To achieve a homogenous adsorption, the mixture was exposed to an ultraturax-generated vortex during the addition of the peptide to GO. The HPLC analysis of collected supernatant from washing process demonstrates the absence of free PMB (Figure S2). The complex was subsequently freeze-dried to maintain its structure and prevent peptide release in the solution during storage. After redispersion of the complex in an aqueous solution, its colloidal stability was reduced compared to GO alone (Figure S3).

To improve the stability of the dispersion, GO was modified by covalent functionalization with a short polyethylene glycol (PEG) chain to promote the dispersion of materials and particles in an aqueous environment. The primary amines of $\text{NH}_2(\text{CH}_2\text{CH}_2\text{O})_2\text{CH}_2\text{CH}_2\text{NH}_2$ (TEG) were allowed to react with the epoxide rings present onto the surface of GO (Figure 1). This approach of GO functionalization is an easy and practical method to reach a high level of functional groups under mild conditions. Verification and quantification of the amino functions were performed after the purification of GO-TEG by dialysis using the Kaiser test. The concentration of the amines resulted in 442 μ mol/g of GO. The order of magnitude is similar to that calculated by XPS (577 μ mol/g of GO) (*vide infra*). Then, PMB was adsorbed onto the surface of GO-TEG under the same condition used for non-functionalized GO. TEG functionalization considerably increased the complex dispersion in an aqueous solution (Figure S3). In addition to HPLC, the different complexes were also characterized by TGA, XPS, and FTIR.

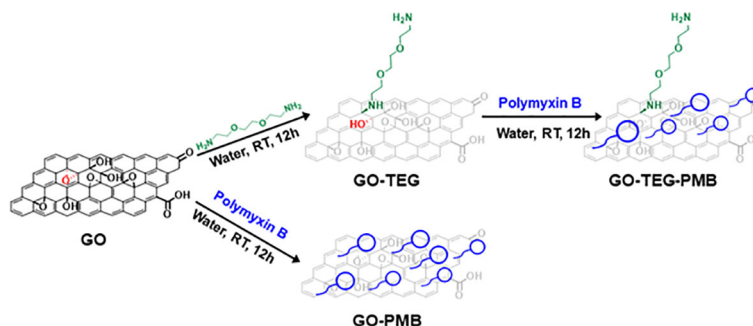


FIGURE 1

Schematic illustration of the processes to prepare GO-based materials combined with PMB. PMB is complexed to the two GO-based materials either after functionalization of GO with the TEG linker or directly to GO.

TGA, conducted under an inert atmosphere, allowed us to evaluate the thermal degradation profile of GO conjugates (Figure 2, left). In the temperature range between 30 and 800°C, GO conjugates lost more weight than pristine GO. Calculation on the derivative thermogram (Figure 2, right) revealed that GO exhibits the typical two-step degradation profile, with a first modest step at around 100°C and a second one at around 200°C, associated with the loss of water and labile oxygenated groups, respectively. After the loss of water and labile oxygenated groups, an additional step could be observed between 300–400°C, depending on the type of functionalities added to the material surface. This additional step in the thermal profile can be attributed to polymer degradation for GO-TEG and polypeptide PMB degradation for GO-PMB and GO-TEG-PMB, respectively. In addition, there is also a noticeable shift in the step of degradation around 200°C for materials containing TEG chains (Figure 2, right). This shift is caused by a change in the surface chemistry of GO caused by the covalent functionalization, which opened the epoxide ring and introduced secondary amines (less thermally stable). The different functionalization steps increased the weight of the compounds, which translates into a greater loss of mass observable above 450°C for the different conjugates, following this trend: GO-TEG (+2.2% compared to GO), GO-PMB (+2.8% compared to GO) and GO-TEG-PMB (+3.3% compared to GO-TEG).

Subsequently, XPS was performed to study the surface composition and chemical state of the different GO conjugates before and after the complexation with PMB (Figure 3). The addition of the TEG or PMB resulted in clear changes in the XPS spectra, as expected. In comparison to the pristine GO, which was mostly composed of C and O atoms, the addition of TEG and/or PMB increased the number of N atoms. The N1s spectra of functionalized GO show two peaks, at 399.6 and 401.6 eV, corresponding to amines and positively charged amines, respectively. In the case of the high-resolution C1s peaks of the different conjugates, the contribution of C–O bonds is reduced in comparison to pristine GO, implying that the introduction of TEG chain or the peptide increases the ratio of C–C bonds. Furthermore, in comparison to the initial GO, a novel contribution to the C1s deconvolution peak at 286.0 eV was identified. Even if this contribution overlaps with the value of the C–OH binding energy, the appearance of this one is caused by the formation of the bond C–N on the surface during the opening of the epoxides by the primary amine of the chain TEG, and by the presence of the PMB, which contains many amide bonds and the unnatural amino acid 2,4-diaminobutyric acid. The analysis of the XPS surveys allowed us to calculate the number of functional groups. The initial content of nitrogen on GO was 0.9%, and the addition of PMB and TEG considerably augmented this amount (Table at the bottom of

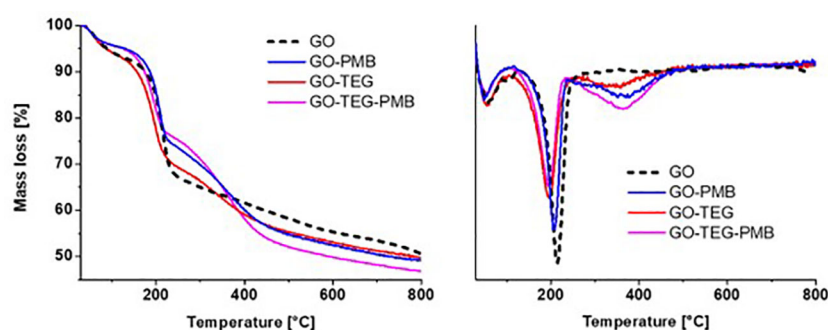


FIGURE 2

Left) Thermogravimetric analysis of GO, GO-PMB, GO-TEG, and GO-TEG-PMB. Right) Derivative thermogravimetry of GO, GO-PMB, GO-TEG and GO-TEG-PMB. The differences in the curves of the weight loss and the shifts in the position of derivative minima indicate the occurrence of the functionalization of GO and the complexation with PMB.

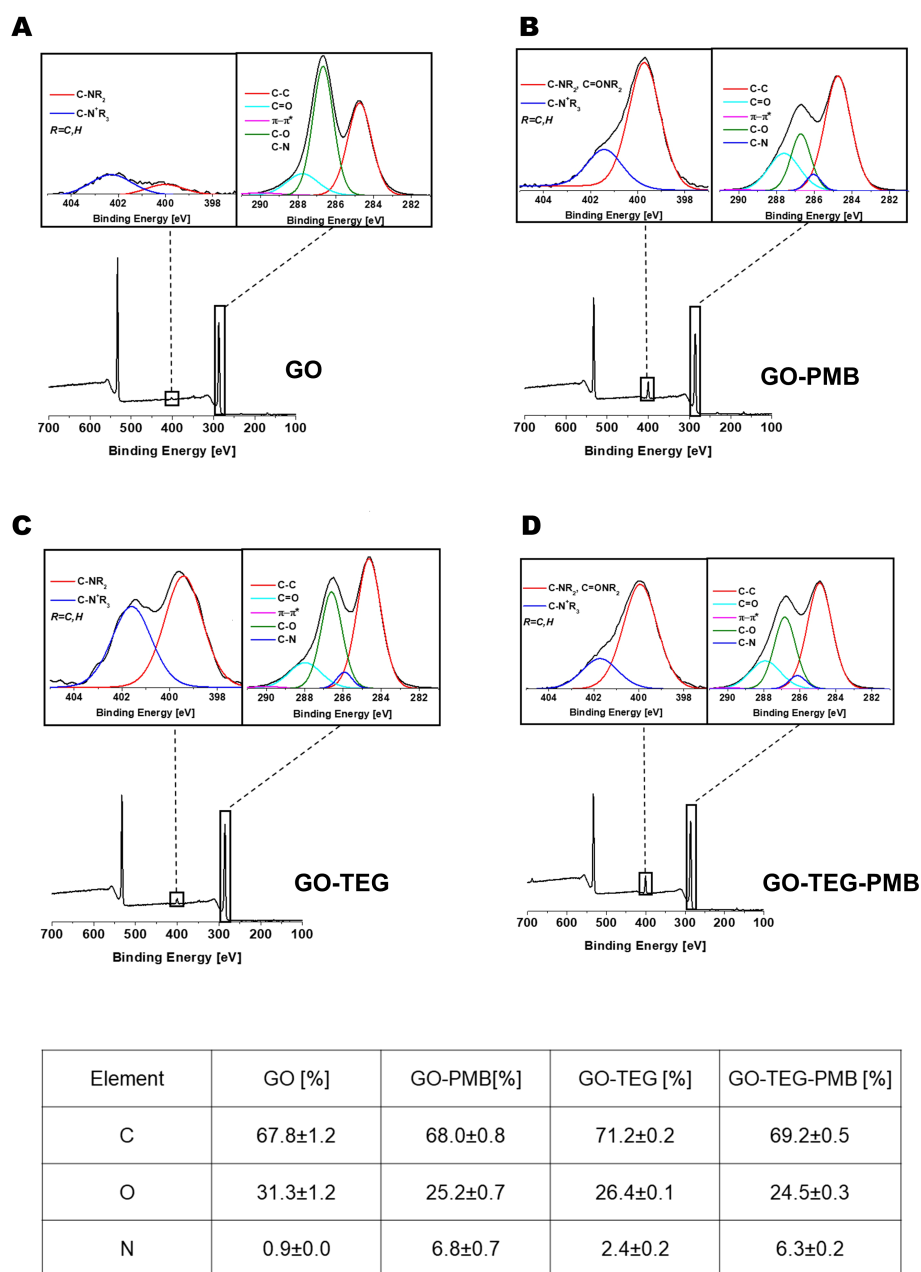


FIGURE 3

XPS characterization. XPS survey spectra with high resolution N1s (left inset) and C1s (right inset) of GO (panel (A)), GO-PMB (panel (B)), GO-TEG (Panel (C)) and GO-TEG-PMB (Panel (D)). Bottom: table reporting the relative XPS atomic percentage of C, O, and N.

Figure 4). Considering the molecular weight of each function, the number of N atoms in the molecules, and the value obtained from the starting GO, the loading of TEG was calculated to be 577 $\mu\text{mol/g}$ of GO (8.5 wt%) for GO-TEG. The loading of PMB corresponded to 281 $\mu\text{mol/g}$ of GO (39 wt%) for GO-PMB, and 228 $\mu\text{mol/g}$ of GO (32 wt%) for GO-TEG-PMB.

Finally, we analyzed the FT-IR spectra of the different GO conjugates to confirm the covalent functionalization with TEG as well as the coupling with PMB (Figure S4). In the spectrum of the starting GO, the broad peak around 3400 cm^{-1} was assigned to the O-H stretching of the adsorbed water and the hydroxyl functions of

GO. The band at 1723 cm^{-1} in GO was attributed to the stretching of C=O groups, while the peak at 1619 cm^{-1} corresponded to the H-O-H bending vibration of water molecules and the skeletal C=C bond vibrations of the graphitic domains. The band at 1371 cm^{-1} could be assigned to the O-H bending vibration. The C-O-C vibration band of the epoxides was located at 1232 cm^{-1} and the peak at 1143 cm^{-1} was assigned to the C-O stretching. The presence of amide I and amide II bands in conjugated GO-PMB and GO-TEG-PMB supports the existence of adsorbed PMB onto the surface of GO. The two peaks located at 1647 cm^{-1} and 1533 cm^{-1} in free PMB can also be found in GO-PMB and GO-TEG-PMB.

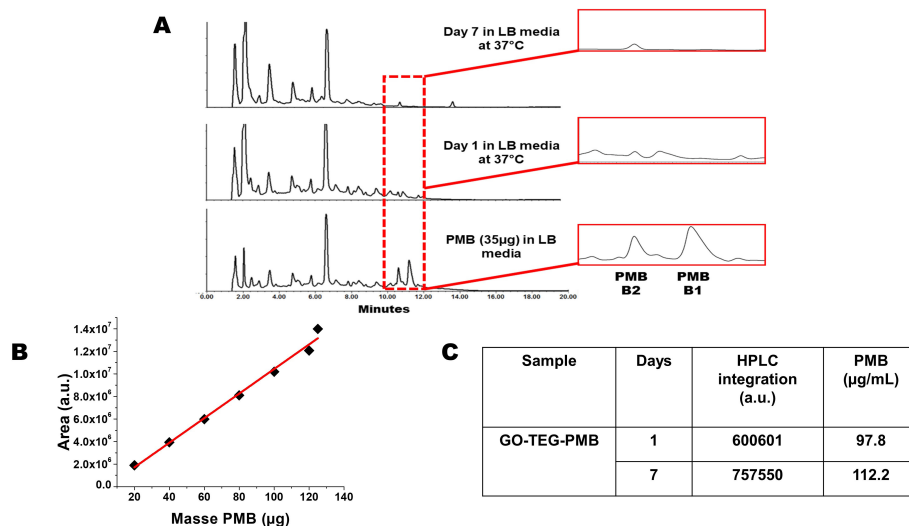


FIGURE 4

(A) HPLC chromatograms of PMB and GO-TEG-PMB stability in LB culture media incubated for 1 day and 7 days at 37°C. (B) Calibration curve using different amounts of PMB injected in HPLC. (C) Table reporting the different integrated areas of PMB peaks released from GO-TEG-PMB on day 1 and day 7.

3.2 Release of PMB from functionalized GO

Our choice of non-covalent complexation between GO and PMB can raise the issue of uncontrolled release of the peptide during the dispersion in the cell culture media and incubation with bacteria. In this context, we investigated the stability of our GO-TEG-PMB conjugate over time. The material was dispersed in LB culture media using a sonication bath, to prepare two dispersions of 500 µL at 2 mg/mL of GO-TEG-PMB. The dispersions were placed in the incubator at 37°C. The first mixture was centrifuged after 1 day and the second one after 7 days. The supernatants were collected and analyzed by HPLC (Figure 4A). A calibration curve with PMB at different concentrations in LB medium was performed (Figure 4B) to calculate the quantity of PMB released over time (Figure 4C). The chromatogram peaks between 10 and 12 min correspond to PMB identified in LB medium. The first belongs to PMB B2 and the second to PMB B1. The two compositions of PMB differ by the terminal fatty acid chain, with PMB B2 possessing a methyl group, while in PMB B1 the analogue chain is an ethyl group. The presence of PMB B2 in the medium is also visible in the chromatograms of the two supernatants at day 1 and day 7. The analogue with the longer fatty acid chain (B1) seems to remain adsorbed more strongly onto the surface of GO than PMB B2, which is mainly released after 24 h. The calculations allowed to establish a release of 98 µg per 1 mg of GO-TEG-PMB after 24 h and

112 µg after 7 days, corresponding to 10% and 11% of release, respectively. On the basis of these data, we can conclude that almost all PMB B2 is rapidly detached from the surface of GO by comparing the ratios of PMB B1 and PMB B2 (3:1), as well as the previously estimated loading (0.32 g of PMB for 1 g of conjugate).

3.3 PMB adsorption significantly enhances the bacteriostatic and bactericidal efficiency of GO

The antimicrobial efficacy of GO and PMB-incorporated GO was first characterized by MIC and MBC values against planktonic *E. coli* and *P. aeruginosa*. As shown in Table 1, the MIC and MBC of pristine GO and TEG functionalized GO were > 100 µg/mL, hence we did not document any antimicrobial effect of GO alone (Table 1). Bacteriostatic and bactericidal efficiency was established after PMB adsorption. The bacteriostatic effect of GO-PMB was stronger than GO-TEG-PMB against *P. aeruginosa*. There was no difference in the bactericidal efficiency of GO-PMB and GO-TEG-PMB against both tested bacterial strains (Table 1).

Based on MIC and MBC values, concentrations of test agents were selected for time kill assays gain more detailed insight into the antimicrobial effect. GO and GO-TEG did not fully inhibit the

TABLE 1 MIC and MBC of GO, GO-TEG, GO-PMB and GO-TEG-PMB against *P. aeruginosa* and *E. coli*.

P. aeruginosa			E. coli		
Material	MIC (µg/mL)	MBC (µg/mL)	Material	MIC (µg/mL)	MBC (µg/mL)
GO	> 100	> 100	GO	> 100	> 100
GO-TEG	> 100	> 100	GO-TEG	> 100	> 100
GO-PMB	25	50	GO-PMB	50	50
GO-TEG-PMB	50	50	GO-TEG-PMB	50	50

growth of *E. coli* and *P. aeruginosa*, but the cell concentration was 10 fold lower than the untreated controls after 24 h incubation (Figure 5). By contrast, the treatment with GO-TEG-PMB significantly reduced the number of viable bacterial cells already after 4 h of incubation. The concentration-dependent bactericidal activity of GO-TEG-PMB was also clearly visible in the time killing assay after 24 h of treatment. Consistent with the MIC and MBC results, *P. aeruginosa* was more sensitive to the tested agents than *E. coli*. The viability of *P. aeruginosa* was drastically decreased by GO-PMB (3 log CFU) and GO-TEG-PMB (5 log CFU) after 24 h of treatment at 2×MIC concentration of tested agents (Figure 5A). In comparable conditions, the viability of *E. coli* was reduced only by 1 and 2 log of CFUs, respectively, by GO-PMB and GO-TEG-PMB (Figure 5B).

3.4 GO-TEG-PMB treatment destroys preformed bacterial biofilms

In order to determine the antibiofilm activity, preformed 24 h old biofilms of *E. coli* and *P. aeruginosa* were treated with all the tested materials. After the 24 h of treatment with each tested agent,

antibiofilm activity was examined by means of CFU counting, live/dead viability staining, and scanning electron microscopy (Figure 7). The viability of both *E. coli* and *P. aeruginosa* biofilms were not significantly affected by GO and GO-TEG. By contrast, GO-PMB and GO-TEG-PMB significantly altered the viability of biofilms of both tested strains. Furthermore, the reduction of a number of biofilm cells was observed to be concentration dependent, since 2×MIC reduced the viability of bacteria significantly higher than 1×MIC (Figure 6A). The antibiofilm effect of GO-TEG-PMB was significantly stronger than that of GO-PMB. Interestingly, the increase of the GO-TEG-PMB concentration to 2×MIC was more effective against the *E. coli* biofilms. This does not follow the trend observed with planktonic bacterial cultures. This difference is likely due to the difference in density and composition of the biofilms matrix of respective bacterial strains, reflecting on their resilience. To confirm the results obtained from CFU counting, control, and treated biofilms were stained with live/dead viability staining and examined using a fluorescence microscope. Figure 6B shows the representative images acquired after the live/dead staining where green color depicts the live cells and red color depicts the dead cells. A higher density of dead cells was observed in biofilms treated with 2×MIC of GO-PMB and GO-TEG-

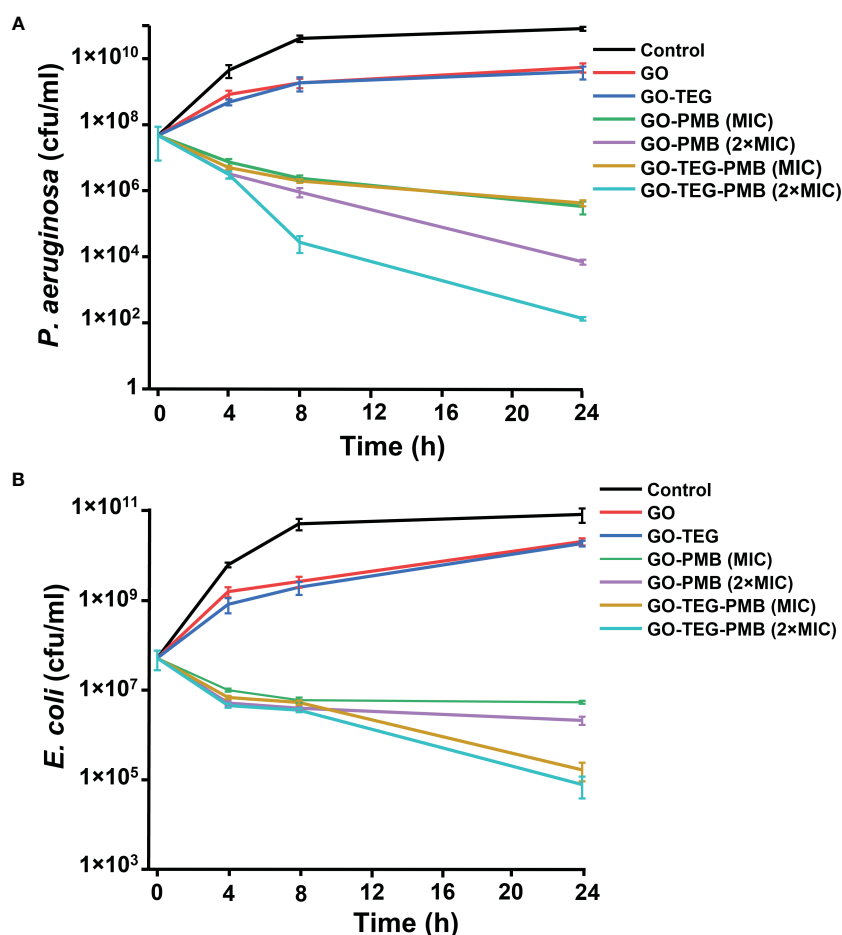


FIGURE 5

Time kills assay of tested agents against the planktonic culture of *E. coli* and *P. aeruginosa*. CFU counts of (A) *P. aeruginosa* and (B) *E. coli* exposed to GO (100 µg/mL), GO-TEG (100 µg/mL), GO-PMB, and GO-TEG-PMB with respect to treatment time. Both bacterial strains are grown in standard cultivating conditions. Data are presented as mean ± standard deviation from three independent biological replicates.

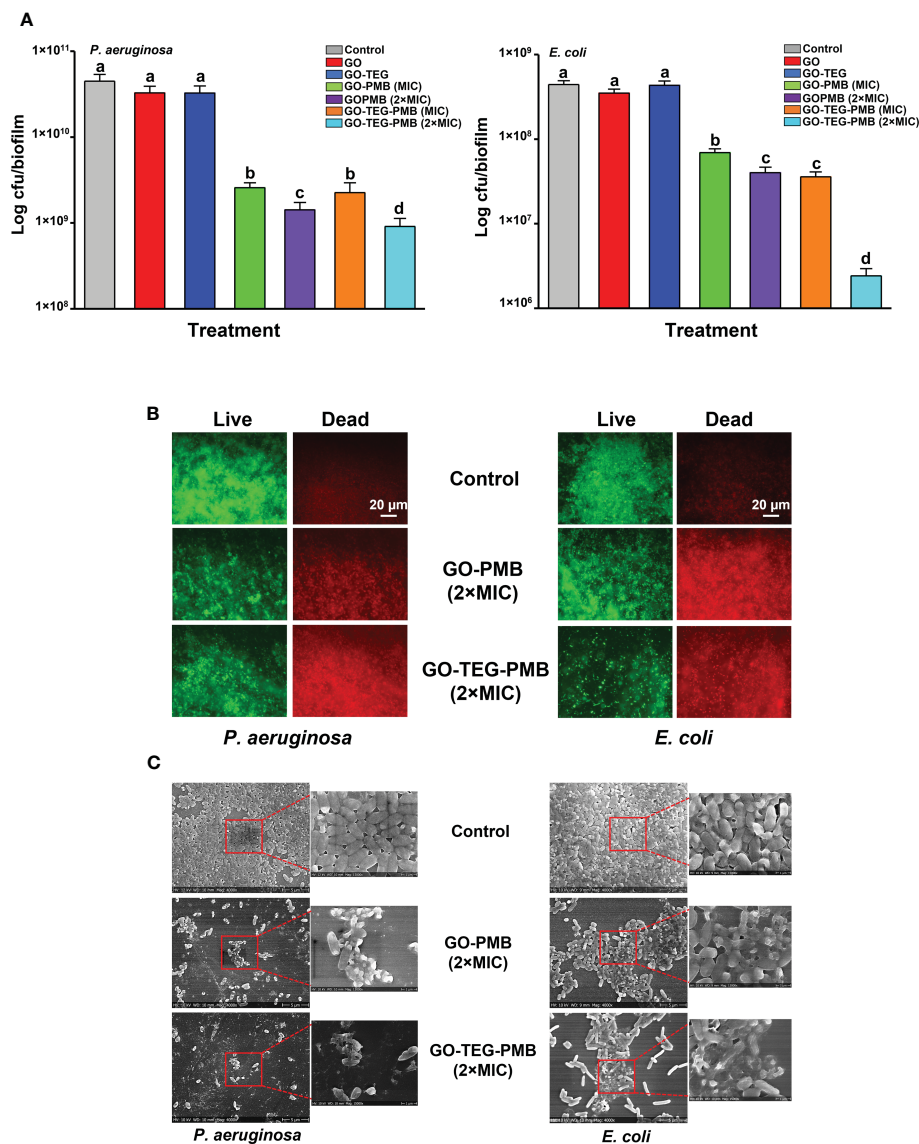


FIGURE 6

Measurement of antibiofilm activity of GO, GO-TEG, GO-PMB, and GO-TEG-PMB against (A) *P. aeruginosa* and *E. coli*. 24 h old biofilms were exposed to fresh medium containing test agents for an additional 24 h, and the viability of bacteria was expressed as CFUs. Data represents mean $\pm$ standard deviation from three independent biological replicates. Values labeled by the different superscript are significantly different from each other ($P < 0.05$). (B) Live/dead viability staining of biofilms of *P. aeruginosa* and *E. coli* after 24 h of treatment. Representative fluorescence microscopy images from three independent biological replicates are presented. Green color denotes live bacteria and red color denotes dead bacteria. (C) Representative SEM images of *P. aeruginosa* and *E. coli* biofilms after 24 h of treatment.

PMB compared to controls. Both *P. aeruginosa* and *E. coli* biofilms were more strongly affected by GO-TEG-PMB (green to red ratios should be compared). These observations correlated well with the findings from CFU counting. Furthermore, to examine the effect of the treatment on the architecture of the biofilm matrix and cell morphology, biofilms were examined by SEM (Figure 6C). Both GO-PMB and GO-TEG-PMB were found to significantly alter the biofilm organization and deactivate bacterial cells by disrupting the cell membranes. Both tested materials at a 2×MIC clearly destroyed the biofilms of both *P. aeruginosa* and *E. coli*, leaving either a fraction of altered microcolonies or single cells disconnected from the complex community. Most of the visible cells in such microcolonies or individual cells were either morphologically altered

or collapsed due to cell membrane damage leading to leakage of intracellular components.

3.5 GO-TEG-PMB coating strongly mitigates biofilm formation on commercial catheters

Next, we asked whether the strong biofilm disruptive activity of GO-TEG-PMB can be useful in terms of preventing biofilm formation on typical biomedical devices. To test that, small pieces of catheter tubes were coated with individual test materials. Representative photographs of test agent coated catheters are

presented in Figure 7A. The coated catheters were exposed to bacterial cultures (24 h) for biofilm formation and the adhesion of bacterial cells on the catheter surfaces was examined by SEM (Figure 7B). As shown in SEM images, bacterial adhesion and biofilm formation of both *P. aeruginosa* and *E. coli* on the surface of pristine GO- and GO-TEG-coated catheters appear to be similar to the control non-coated surface. By contrast, bacterial adhesion was drastically reduced on catheters coated with GO-PMB and GO-TEG-PMB. In addition to preventing bacterial adhesion, GO-PMB and GO-TEG-PMB coatings seemed to also disrupt the adhered bacterial cells.

4 Discussion

The goal of this study was to improve the efficacy of GO as an antibacterial agent against Gram-negative biofilms. To achieve this, several aspects were taken into consideration. One of the strategies is based on a combined antibacterial effect of GO and PMB. Previous studies have reported that the lateral size of GO should be around one micrometer (Liu et al., 2011; Liu et al., 2012) for

optimal intrinsic effectiveness of the material against bacteria. GO sheets possessing this lateral size can fully cover the bacterial membrane by wrapping the cells, thus inhibiting bacterial metabolic activity and isolating them from the surrounding environment (Akhavan et al., 2011). However, size is not the only factor to consider when designing antibacterial GO sheets. Another parameter to consider is the level of oxidation. According to the strategy we adopted for our study, it was preferable to choose a highly oxidized GO, to allow for better molecular adsorption (Thangavel and Venugopal, 2014) and to boost the oxygenated functionalities for covalent modification of the surface. PMB was chosen because it is an effective antimicrobial agent used against multidrug-resistant bacteria (Zavascki et al., 2007; Siddiqui et al., 2014). We decided to complex pristine and functionalized GO with the antimicrobial peptide PMB. Since GO contains graphitic sp^2 domains and areas rich in oxygenated functions (e.g., hydroxyl and epoxy groups), physisorption between GO and PMB can occur via π - π stacking, electrostatic forces, and hydrogen bonding.

In this study, PMB was mixed with GO and TEG functionalized GO in order to achieve adsorption onto surface of pristine and functionalized GO. The rationale of TEG functionalization was to

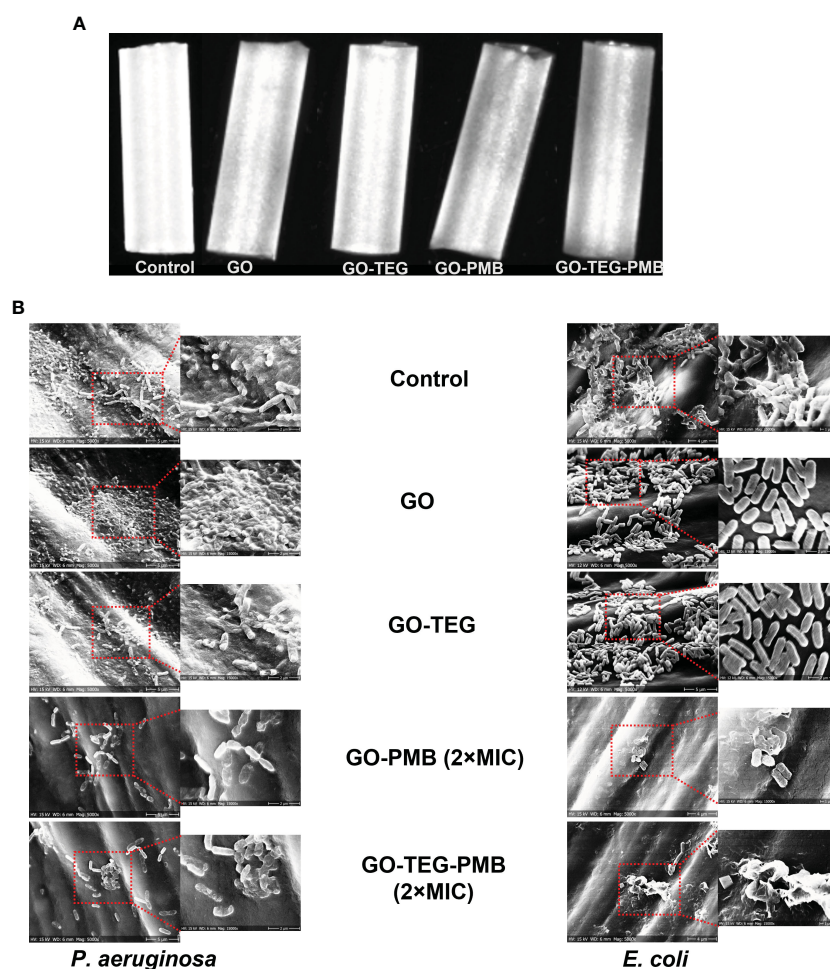


FIGURE 7

(A) Photograph of GO, GO-TEG, GO-PMB and GO-TEG-PMB coated polymer-based catheter tubes. (B) Representative scanning electron microscopy images of *P. aeruginosa* and *E. coli* biofilm formation on coated and non-coated catheter tubes after 24 h of bacterial cultivation.

add a hydrophilic chain to enhance the dispersibility of GO. The adsorption of PMB was confirmed by XPS and FTIR (Figure 3, Figure S4) and HPLC results confirmed gradual release of PMB from the complex material, with about 10% release after one day, and further 10% release up to day 7 (Figure 4). This indicated that coatings based on GO-TEG-PMB could provide sustained delivery of PMB and offer long-term antibacterial protection. From the physicochemical analyses of the complexes we can draw the following conclusions. The improvement in the dispersibility of the complex resulting from the mixture of GO and PMB was achieved through the insertion of a TEG chain via the formation of covalent bonds. This covalent linking occurs via the ring opening of the epoxides onto the surface of GO by the primary amine of TEG as demonstrated in previous articles (Vacchi et al., 2016). The interactions between the two GO materials and PMB are mainly electrostatic and hydrophobic, the latter likely due to the presence of the lipid chain on PMB, while π - π interactions are unlikely as there is only one phenylalanine in the peptide sequence. Indeed, we did not observe an increase of the π - π signals in the high resolution XPS C1s spectra.

The antimicrobial activity of the engineered materials was then evaluated by MIC and MBC assays. As expected, PMB adsorption brought down the MIC and MBC concentration of GO to 25 and 50 μ g/mL (Table 1), respectively, suggesting the increase in antimicrobial potential. Time dependent killing assays further confirmed the bacteriostatic and bactericidal efficiency of pristine and PMB adsorbed GO. The strong time dependent bactericidal activity of GO-TEG-PMB can be due to the higher release of PMB compared to GO-PMB (Figures 4C, 5). Despite exhibiting a similar bactericidal trend against both *P. aeruginosa* and *E. coli*, the killing efficiency of GO-TEG-PMB was higher against *P. aeruginosa*. This difference is most likely due to the strain difference in sensitivity to PMB and GO.

Most of the previous studies regarding the antimicrobial activity of pristine and functionalized GO were conducted with bacterial cells in a planktonic state (de Moraes et al., 2015; Karahan et al., 2016; Ye et al., 2017). However, in clinical as well as environmental settings, bacterial cells tend to adhere to surfaces and form 3-dimensional structure called biofilms (Kordmahaleh & Shalke, 2013). The bacterial communities in such biofilm matrix tolerate up to 1000 times higher concentrations of antimicrobial agents (Sharma et al., 2019). It is important to consider testing the potential of drug/nanomaterials against the biofilm cells if the agents are aimed at the treatment of infectious diseases. Thus, to examine antibiofilm potential, preformed biofilms were treated with GO, GO-PMB, and GO-TEG-PMB. The obtained results clearly demonstrated a concentration-dependent bactericidal efficiency of the tested materials against biofilms. Since pristine GO and GO-TEG showed no bactericidal activity, we would propose that the observed antibiofilm activity of GO-PMB and GO-TEG-PMB is mainly due to the release of PMB in contact with the biofilms. This difference in antibiofilm activity against different bacterial strains may be due to the difference in the composition and density of exopolymer matrix in the biofilms of *E. coli* and *P. aeruginosa*.

Furthermore, the results from anti-biofilm activity of PMB adsorbed GO were confirmed by live/dead viability staining. The staining kit contains two nucleic acid stains SYTO 9 and propidium iodide. The fluorescence stain SYTO 9 can permeate the cell membrane, thus entering living cells and staining them in green. Propidium iodide cannot permeate the cell membrane, hence only the cells that are damaged and morphologically altered and stained in red. As presented in Figure 6B, there is an increase in the density of dead cells in the biofilms treated with GO-PMB and GO-TEG-PMB. While the live/dead fluorescence microscopy images can visualize the general trend of bactericidal efficiency of tested agents against biofilms, it cannot clearly visualize the intensity of damage to cells inside the biofilm matrix. To visualize this, treated biofilms were examined with SEM (Figure 6C). As suggested by the number of propidium iodide-stained cells, the treatment led to extensive damage or full collapse of the cell membranes in most of the visualized cells.

Prevention of biofilm formation is considered as an excellent approach for inhibition of biomedical device associated infections. Indeed, the adhesion of pathogenic microorganism to biomedical devices and subsequent biofilm formation is a great challenge and may cause severe infections (Lebeaux et al., 2014). Antimicrobial coatings using nanomaterials and composites are considered as one of the promising approaches to prevent biofilm formation on the surface of such devices (Pandit et al., 2018; Chen et al., 2020). To test the efficiency of PMB adsorbed GO in preventing biofilm formation, catheter tubes were coated with GO-TEG-PMB. This has led to a very clear reduction of bacterial adhesion and visible damage to the attached cells. These results suggested that coatings based on GO-TEG-PMB are truly applicable in clinical settings, with a clear potential for preventing biofilm formation on medical devices. In summary, we propose that our method for combining GO and PMB can lead to a material that is easy to produce, easy to apply as a coating on biomedical devices and offers strong and lasting protection against Gram-negative bacterial biofilms based on the sustained release of the antibacterial agent.

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.

Author contributions

Conceptualization, SP, IM, AB, and CP. Methodology, SP, LJ, JZ, ZG, YN, and CP. Validation, SP, LJ, JZ, ZG, YN, and CP. Data curation, SP, LJ, JZ, ZG, YN, and CP. Writing—original draft preparation, SP, LJ, and CP. Writing—review and editing, SP, RM, IM, AB, and CP. Project administration, SP, IM, AB, and CP. Funding acquisition, SP, CP, IM, and AB. All authors contributed to the article and approved the submitted version.

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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/fcimb.2023.1209563/full#supplementary-material>

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Monoclonal antibodies against lipopolysaccharide protect against *Pseudomonas* *aeruginosa* challenge in mice

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Pseudomonas aeruginosa is a common cause of hospital-acquired infections, including central line-associated bloodstream infections and ventilator-associated pneumonia. Unfortunately, effective control of these infections can be difficult, in part due to the prevalence of multi-drug resistant strains of *P. aeruginosa*. There remains a need for novel therapeutic interventions against *P. aeruginosa*, and the use of monoclonal antibodies (mAb) is a promising alternative strategy to current standard of care treatments such as antibiotics. To develop mAbs against *P. aeruginosa*, we utilized ammonium metavanadate, which induces cell envelope stress responses and upregulates polysaccharide expression. Mice were immunized with *P. aeruginosa* grown with ammonium metavanadate and we developed two IgG2b mAbs, WVDC-0357 and WVDC-0496, directed against the O-antigen lipopolysaccharide of *P. aeruginosa*. Functional assays revealed that WVDC-0357 and WVDC-0496 directly reduced the viability of *P. aeruginosa* and mediated bacterial agglutination. In a lethal sepsis model of infection, prophylactic treatment of mice with WVDC-0357 and WVDC-0496 at doses as low as 15 mg/kg conferred 100% survival against challenge. In both sepsis and acute pneumonia models of infection, treatment with WVDC-0357 and WVDC-0496 significantly reduced bacterial burden and inflammatory cytokine production post-challenge. Furthermore, histopathological examination of the lungs revealed that WVDC-0357 and WVDC-0496 reduced inflammatory cell infiltration. Overall, our results indicate that mAbs directed against lipopolysaccharide are a promising therapy for the treatment and prevention of *P. aeruginosa* infections.

KEYWORDS

Pseudomonas aeruginosa, monoclonal antibody (mAb), anti-lipopolysaccharide antibody, O5, ammonium metavanadate, sepsis, pneumonia, immunotherapeutics

1 Introduction

Pseudomonas aeruginosa is a ubiquitous, Gram-negative pathogen that thrives in many natural environments and habitats (Diaz et al., 2018; Laborda et al., 2021). As part of the pseudomonad genus, *P. aeruginosa* has minimal nutritional requirements and can persist on frequently contacted surfaces found in hospital rooms and medical equipment (Pachori et al., 2019). Consequently, *P. aeruginosa* is one of the most common sources for nosocomial infections and can cause serious illness in immunocompromised patients, such as ventilator-associated pneumonia (VAP) and primary bloodstream infections (Spagnolo et al., 2021). Among Gram-negative bacteria, *P. aeruginosa* is the most common cause of VAP (Sader et al., 2014), and second most common cause of primary bloodstream infections (Richards et al., 1999). The healthcare and socioeconomic burden from VAP and primary bloodstream infections are significant, and these complications are associated with increased mortality, greater hospital costs, and longer lengths of stay (Chastre and Fagon, 2002; Brun-Buisson et al., 2003; Warren et al., 2003; Wisplinghoff et al., 2004; Chiang et al., 2021).

Although the selection of appropriate antibiotics is central in improving patient outcomes, the development of antibiotic resistance by *P. aeruginosa* has presented an enormous challenge for healthcare providers. *P. aeruginosa* can develop resistance to multiple classes of antibiotics through intrinsic and acquired mechanisms of resistance, which include multi-drug efflux pumps, antibiotic-modifying enzymes, and the horizontal transfer of mobile genetic elements (Pang et al., 2019). Of concern is the prevalence of multidrug-resistant (MDR) *P. aeruginosa* (resistance to three or more drug classes). In a review by Puzniak et al., MDR *P. aeruginosa* was accountable for 72.3% of all *P. aeruginosa* infections (Puzniak et al., 2021). In 2017, the CDC reported that infections from MDR *P. aeruginosa* were responsible for 32,600 hospitalizations, 2,700 deaths, and \$767 million in attributable healthcare costs (Centers for Disease Control, 2019). As a result, MDR *P. aeruginosa* has been listed as a serious public health threat that requires immediate action (Centers for Disease Control, 2019). Although there is a need for new therapeutic agents against MDR *P. aeruginosa*, there are few drugs in the developmental pipeline (Butler et al., 2022).

An important virulence factor in the pathogenesis of *P. aeruginosa* infections is lipopolysaccharide (LPS), which promotes evasion of host defenses and establishment of infection (Huszczynski et al., 2020). LPS is also a potent stimulator of the innate immune system and can generate strong inflammatory responses. This host response is mediated by the activation of toll-like receptor 4 (TLR4), which triggers production of pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α (Kawai et al., 2001). However, if left unregulated, an excessive production of pro-inflammatory cytokines can eventually lead to severe inflammatory conditions, such as sepsis and multi-organ failure (Ulloa and Tracey, 2005; Kell and Pretorius, 2015). The passive administration of monoclonal antibodies (mAbs) can provide rapid protection against *P. aeruginosa* infections, and is ideal for susceptible individuals who are immunocompromised or have acute exposure to the pathogen (Krause et al., 1997). Numerous

pre-clinical studies have demonstrated that the administration of anti-LPS mAbs against the O-antigen of *P. aeruginosa* reduces bacterial burden and improves survival in multiple animal models of infection (Akiyama et al., 2000; Hemachandra et al., 2001; Horn et al., 2010; Xie et al., 2010). In a phase IIa clinical trial with Panobacumab, an anti-LPS mAb against serotype O11, it was shown that adjunctive therapy with standard of care antibiotics improved clinical resolution of patients with confirmed O11 pneumonia (Que et al., 2014). Though there are currently no FDA approved mAbs for *P. aeruginosa* infections, the promising outcomes of these pre-clinical and clinical studies support the further development of anti-LPS mAbs against *P. aeruginosa*.

One method for upregulating bacterial cell surface polysaccharides is to use ammonium metavanadate (AMV), which is a phosphatase inhibitor that induces cell envelope stress responses (Damron et al., 2011). When *P. aeruginosa* is cultured on *Pseudomonas* isolation agar (PIA) supplemented with AMV (PIAAMV), numerous genotypic changes occur, and the resultant phenotype closely resembles other clinical isolates that have undergone similar envelope stress responses. Notably, we had previously demonstrated that when *P. aeruginosa* PAO1 was cultured on PIAAMV, a large number of genes associated with LPS increased expression levels, including the entire O-antigen operon (*wzz* to *wbpM*) (Damron et al., 2013). In addition, the lipid A moiety was altered to include a palmitate group (Damron et al., 2011), which is a common modification among cystic fibrosis isolates (Ernst et al., 2007). This hexa-acylated form of lipid A has been shown to increase recognition of TLR4 by *P. aeruginosa* (Hajjar et al., 2002). Interestingly, in a survival study of mice challenged with *P. aeruginosa* PAO1 cultured on PIA or PIAAMV, there was an improved survival of mice in the PIAAMV group (Damron et al., 2013). Based on these findings, we hypothesized that the predominant antibody response in mice challenged with *P. aeruginosa* cultured on PIAAMV was likely to outer membrane polysaccharides and the development of these antibodies would lead to a protective therapy against *P. aeruginosa* infections.

Herein, we describe the discovery of mAbs that are highly protective against *P. aeruginosa* infections. WVDC-0357 and WVDC-0496 were derived from mice challenged with *P. aeruginosa* PAO1 cultured on PIAAMV. We demonstrate that these mAbs are highly protective in clinically relevant models of sepsis and acute pneumonia. Our data demonstrate that WVDC-0357 and WVDC-0496 reduce the number of viable bacteria and protect against *P. aeruginosa* challenge in multiple models of infection. Furthermore, protection provided by WVDC-0357 and WVDC-0496 was linked with a decreased inflammatory cytokine profile. Altogether, these results support the translation and use of WVDC-0357 and WVDC-0496 against *P. aeruginosa* infections.

2 Materials and methods

2.1 Generation of hybridomas

Six-week-old CD-1 mice (Charles River, strain 022) were primed and boosted with an intranasal (IN) dose of 1×10^7 CFU

of live *P. aeruginosa* strain PAO1 Vasil (Barton et al., 1996) grown on Pseudomonas isolation agar (PIA) supplemented with 0.3 mM ammonium metavanadate (AMV). After 60 d, mice were euthanized and the spleen was harvested for hybridoma generation. Hybridomas were generated by fusing 1×10^7 splenocytes with 1×10^7 SP2/O-Ag14 myeloma cells (ATCC CRL-1581TM) using the ECM2001+ electrofusion apparatus (BTX). Fused cells were cultured in ClonaCell-HY medium C (STEMCELL Technologies) and incubated overnight at 37°C and 5% CO₂. The next day, cells were centrifuged and cultured in ClonaCell-HY medium D (STEMCELL Technologies), which is a semi-solid medium containing hypoxanthine-aminopterin-thymidine (HAT). After ten days of incubation, single hybridoma colonies were selected and plated in 96-well plates containing ClonaCell-HY medium E (STEMCELL Technologies). Supernatant from the well plates were screened with ELISA to identify IgG antibodies against *P. aeruginosa*. Select hybridoma colonies were produced at high densities using a hollow fiber bioreactor (FiberCell Systems) containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with serum-free chemically defined medium for high density cell culture (CDM-HD). Antibodies from the bioreactor harvests were purified with the ÄKTA pure chromatography system (Cytiva) using a HiScreen FibroTM Prism A column (Cytiva) and HiScaleTM 16/20 column (Cytiva). Purified mAbs were eluted in 0.2M sodium phosphate buffer (pH 7.4) and antibody concentrations were determined by measuring the Protein A280 with an E1% value of 14.00. The isotype of each antibody was determined using the Pierce Rapid Antibody Isotyping Kit (ThermoFisher Scientific, #26179).

2.2 Enzyme-linked immunosorbent assay

ELISA assays were performed using a panel of *P. aeruginosa* strains (Supplementary Table 1). *In silico* serogrouping of *P. aeruginosa* strains were performed with whole genome sequences using the *Pseudomonas aeruginosa* serotyper (PAst) (Thrane et al., 2016). *P. aeruginosa* strains were grown on PIA overnight at 37°C. Bacterial plates were swabbed into sterile PBS and adjusted to a bacterial density that corresponded to 1×10^9 CFU/ml. To prepare ELISA plates, 96-well microtiter plates (ThermoFisher Scientific, #15041) were coated with 50 µL of bacteria containing 5×10^7 CFU in phosphate buffered saline (PBS) and incubated overnight at 4°C. Wells were washed three times with PBS with 0.05% Tween 20 (PBS-T) and blocked with 2% bovine serum albumin (BSA) (Research Products International, #A30075) overnight at 4°C. Primary antibodies (serum, hybridoma supernatant, WVDC-0357, or WVDC-0496) diluted in 2% BSA were applied to the wells and incubated for 1 h at 37°C. Wells were washed four times with PBS-T. For plates developed with tetramethyl benzidine (TMB), 100 µL of anti-IgG secondary antibody conjugated to horseradish peroxidase (HRP) (Novus biological, #NBP1-75130) at a dilution of 1:2000 in 2% BSA was applied to each well for 1 h at 37°C. After incubation, wells were washed five times with PBS-T and 100 µL of a TMB solution (BioLegend, #421101) was added to

each well. After 10-15 min, the reaction was stopped by adding 50 µL of 2N sulfuric acid and absorbance was read at 450 nm using a SpectraMax i3 plate reader (Molecular Devices LLC). For plates developed with Pierce p-Nitrophenyl Phosphate (PNPP), 100 µL of anti-IgG secondary antibody conjugated to alkaline phosphatase (AP) (Southern Biotech, #1030-04) at a dilution of 1:2000 in 2% BSA was applied to each well for 1 h at 37°C. After incubation, wells were washed five times with PBS-T and 100 µL of a PNPP solution (ThermoFisher Scientific, #34045) was added to each well. After 30 min, the absorbance was read at 405 nm using a SpectraMax i3 plate reader. Absorbance was plotted against antibody concentration and the area under the curve (AUC) was calculated using Graphpad Prism.

2.3 Immunoblotting

To identify the target of antibody binding, western blot analysis was performed with *P. aeruginosa* strain PAO1 treated with proteinase K or sodium periodate as demonstrated by Nielsen et al. (2017). In addition, western blot analysis was performed with strains from the University of Washington *P. aeruginosa* PAO1 transposon mutant library (Held et al., 2012). Outer membrane fractions were prepared based on methods described by Hernández-Allés et al. (1999). *P. aeruginosa* PAO1 outer membrane fractions were treated with 0.2 mg/ml proteinase K (Invitrogen, #25530049) or with 20 mM sodium periodate (ThermoFisher Scientific, #20504) at 37°C for 2 h. Afterwards, 2 µg of sample was added to Laemmli buffer (Sigma-Aldrich, #S3041) and boiled at 95°C for 5 min. Samples were then loaded into 12% polyacrylamide gels (ThermoFisher, #XP00122BOX) and resolved by gel electrophoresis. Samples were transferred onto nitrocellulose membranes (ThermoFisher, #IB23001) using the iBlotTM 2 Gel Transfer Device (ThermoFisher, #IB21001) and blocked in 5% skim milk overnight. Membranes were treated with 2 µg/ml of WVDC-0357 or WVDC-0496 in 1% skim milk for 1 h at room temperature. Membranes were then washed three times with PBS-T and treated with anti-IgG secondary antibody conjugated to HRP (Novus biological, #NBP1-75130) at a 1:5000 dilution in 1% skim milk for 1 h at room temperature. Membranes were washed three times with PBS-T and developed using PierceTM ECL Western Blotting Substrate (ThermoFisher, #32106). Chemiluminescent images of the blots were taken using a Chemidoc Touch Imaging System (Bio-Rad, #1708370).

2.4 Flow cytometry binding assay

To demonstrate binding of WVDC-0357 and WVDC-0496 to *P. aeruginosa* with flow cytometry, we used methods described previously (DeJong et al., 2022). Briefly, *P. aeruginosa* strain PAO1 was cultured to mid-log phase growth, washed, and concentrated in PBS. Approximately 1×10^7 CFUs of bacteria were incubated with 10 µg/ml of an IgG2b isotype control mAb (BioXcell, #BE0086), WVDC-0357, or WVDC-0496 for 30 min at 37°C. Samples were

washed once with PBS and incubated with 1.25 µg/ml Fc block (BD Biosciences, #553142) for 15 min at 4°C. Afterwards, 2.5 µg/ml of anti-IgG secondary antibody conjugated to APC-Cy7 (BioLegend, #405316) was added to samples and incubated for 30 min at 4°C. Samples were washed twice with PBS and *P. aeruginosa* was stained with BacLight Green bacterial stain (Invitrogen, #B35000). Samples were analyzed on a Guava® easyCyte 12HT flow cytometer (Luminex). Flow cytometry results were analyzed using FlowJo™ v10.8 Software (BD Life Sciences).

2.5 Measurement of antibody binding kinetics

To determine the binding kinetics of WVDC-0357 and WVDC-0496, a flow cytometry binding assay was performed. To allow bacteria-antibody complexes to form, 1×10^7 CFU of *P. aeruginosa* PAO1 was incubated with varying concentrations of an IgG2b isotype control mAb, WVDC-0357, or WVDC-0496 for 1 h at 4°C. Samples were washed once and resuspended in 100 µL of PBS. Afterwards, bacteria were exposed to a 1:400 dilution of rabbit anti-mouse anti-IgG conjugated to Alexa-Fluor 488 (ThermoFisher Scientific, #A11059). Samples were washed once in PBS and fixed in 0.4% v/v paraformaldehyde (Sigma-Aldrich, #P6148) overnight at 4°C. Following fixation, samples were washed twice and resuspended in 300 µL of PBS. Samples were analyzed using a BD Fortessa and results were analyzed using FlowJo™ v10.8 Software. Data were fitted with a non-linear regression fit to determine the dissociation constant (K_D).

2.6 Assessment of bacterial clearance

To assess the capability of WVDC-0357 and WVDC-0496 to facilitate clearance of bacteria, a modified version of an opsonophagocytosis killing assay was performed as described by Paschall et al. (2019). *P. aeruginosa* strain PAO1 was cultured to mid-log phase growth, washed, and resuspended to 1×10^4 CFU/ml in opsonization buffer B (OBB; PBS with Ca^{2+} and Mg^{2+} , supplemented with 5% heat-inactivated fetal bovine serum and 0.1% gelatin). In round bottom 96-well plates (Fisherbrand, #FB012932), 50 µL of bacteria and 50 µL of treatment (PBS, IgG2b isotype control mAb, WVDC-0357, or WVDC-0496 at varying concentrations) were added to each well and incubated for 1 h at room temperature with shaking at 120 rpm. Afterwards, J774A.1 macrophages (ATCC, #TIB-67™), baby rabbit complement (Pel-Freez Biologicals, #31061), J774A.1 macrophages with baby rabbit complement, or OBB was added to each well and incubated for 1 h at 37°C with shaking at 120 rpm. A 1000:1 effector-to-target cell ratio of macrophages to bacterium was used. After incubation, plates were put on ice for 15 min to stop the reaction. Samples were serially diluted and plated on PIA. Plates were incubated overnight at 37°C. Remaining bacterial colonies were enumerated and expressed as percent killings relative to assays run without antibody.

2.7 Assessment of agglutination and turbidity in *P. aeruginosa* cultures

In UV-transparent cuvettes (BrandTech, #759015), an overnight culture of *P. aeruginosa* PAO1 was diluted 1:50 in lysogeny broth and treated 100 µg/ml of IgG2b isotype control, WVDC-0357, or WVDC-0496 mAb. Cuvettes were incubated statically at 37°C and the absorbance at 600 nm was read at time points up to 24 h. To visualize turbidity of growth cultures, cuvettes were photographed at 24 h. To visualize and quantify bacterial agglutination, *P. aeruginosa* PAO1 was cultured to mid-log phase growth, washed, and concentrated in PBS. Approximately 5×10^7 CFUs of bacteria were stained with BacLight Red bacterial stain (Invitrogen, #B35001) for 15 min at room temperature, pelleted, and resuspended in PBS. Antibody was added at a final concentration of 100 µg/ml and incubated statically at 37°C for 2 h. Samples were placed on glass slides, mounted with cover slips, and sealed with nail polish. Fluorescent images were taken using an EVOS FL imaging system (ThermoFisher, #AMEFC4300). For aggregation analysis, 20 randomly imaged areas were analyzed for number and size of aggregates using a custom pipeline in CellProfiler 4.2.4.

2.8 Transmission electron microscopy imaging

To label and visualize external structures on the surface of *P. aeruginosa* with antibodies, we followed an immunogold labeling method described by Javed et al. (2015). Briefly, formvar-coated copper (300 mesh) grids (Sigma-Aldrich, #TEM-FCF300CU50) were floated on drops of an overnight culture of *P. aeruginosa* for 1 h. The grids were washed once in PBS and fixed in 2.5% paraformaldehyde for 20 min. The grids were washed three times in PBS and placed in blocking solution (5% BSA in 0.05% PBS-T). Grids were then incubated with 10 µg/ml of an IgG2b isotype control mAb, WVDC-0357, or WVDC-0496 for 45 min. Grids were washed three times in blocking solution and incubated with goat anti-mouse IgG conjugated to 10 nm gold (Sigma-Aldrich, #G7652) diluted 1:50 in blocking solution for 45 min. The grids were then washed three times in blocking solution, PBS, and milli-Q water. The grids were air dried on Whatman filter paper (Cytiva, #1001085). The grids were examined using a transmission electron microscope (JEOL JEM-2100) equipped with a Gatan OneView camera.

2.9 Murine challenge models

To assess protection in convalescent mice previously challenged with *P. aeruginosa* PAO1 cultured on PIAAMV, an acute pneumonia model was used. *P. aeruginosa* strain PAO1 was cultured on PIAAMV overnight at 37°C. Bacterial plates were swabbed and resuspended in PBS. Based on the optical density at 600 nm, a dose was prepared at 1.4×10^7 CFU per 20 µL in PBS. Six-

week old, female CD-1 mice were anesthetized with ketamine (77 mg/kg) (Patterson Veterinary, #07-803-6637) and xylazine (7.7 mg/kg) (Patterson Veterinary, #07-909-1939) in 0.9% saline. Anesthetized mice were IN challenged by pipetting 10 μ L of bacteria in each nostril. Mice were allowed to convalesce for 34 days. A dose of *P. aeruginosa* PAO1 cultured on PIA was prepared at 1.4×10^8 CFU per 20 μ L. Convalescent mice and age-matched naïve CD-1 mice were anesthetized and IN challenged with the prepared dose. Mice were euthanized at 16 h post-challenge with an intraperitoneal (IP) injection of Euthasol (390 mg/kg) (Patterson Veterinary, #07-805-9296). Blood was collected *via* cardiac puncture and placed in serum separator tubes (BD, #365967). The lung was aseptically removed, homogenized, and plated to determine CFU loads. The nasal cavity was flushed with PBS and plated to determine CFU loads in the nares.

To evaluate the efficacy of WVDC-0357 and WVDC-0496 in a sepsis model of infection, nine-week old female CD-1 mice were IP administered either an IgG2b isotype control mAb, serum from heat-killed *P. aeruginosa* whole-cell vaccinated (*Pa* WCV) mice (Sen-Kilic et al., 2021; Blackwood et al., 2022), WVDC-0357, or WVDC-0496. After 12 h, mice were challenged with an IP 5×10^5 CFU dose of *P. aeruginosa* PAO1. To determine survival, mice were monitored for up to 5 days after challenge. Health of the mice were scored based on six criteria: appearance, activity, eye closure, respiration quality, body temperature, and body weight loss. For each category, mice were scored from 0-4, where 0 represented no symptoms, and 4 represented the most severe phenotype (Shrum et al., 2014). Mice were humanely euthanized if mice reached a score of 4 in any category or a total score of 12 and above.

To look at correlates of protection in the sepsis model, mice were passively immunized as before, and euthanized at 6 h post-challenge for tissue collection. Mice were euthanized with an IP injection of Euthasol (390 mg/kg). Blood was collected *via* cardiac puncture and placed in serum separator tubes. The kidney and spleen were aseptically removed. The right kidney was dounce homogenized. The spleen was homogenized in a gentleMACS C tube (Miltenyi Biotec, #130-093-237). To quantify bacterial burden, blood, kidney, and spleen samples were serially diluted in PBS, plated on PIA, and incubated overnight at 37 °C for CFU enumeration.

To assess the efficacy of WVDC-0357 and WVDC-0496 in an acute pneumonia model, six-week old female CD-1 mice were IP administered either an IgG2b isotype control mAb, *Pa* WCV serum, WVDC-0357, or WVDC-0496. After 12 h, mice were IN challenged with a 2×10^7 CFU dose of *P. aeruginosa* PAO1. Mice were euthanized at 16 h post-challenge with an IP injection of Euthasol (390 mg/kg). Blood was collected *via* cardiac puncture and placed in serum separator tubes. The lung was aseptically removed and weighed. The right lobe of the lung was placed in a gentleMACS C tube and homogenized. The nasal wash was collected by injecting 1 mL of PBS through the nasal cavity. The homogenized lung and nasal wash were serially diluted, plated on PIA, and incubated overnight at 37 °C for CFU enumeration.

All animal care and use procedures in this study was in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory animals. The West Virginia University Institutional Animal Care and Use Committee (WVU-ACUC protocol #1606003173) granted approval for all animal protocols.

2.10 Pharmacokinetics

To assess the pharmacokinetic profile of WVDC-0357 and WVDC-0496, nine-week old CD-1 mice were IP administered WVDC-0357 or WVDC-0496 at 45 mg/kg. There were 3 mice per group. Mice were submandibular bled at 1, 3, 7, 14, and 21 d post-administration of mAb. Sera was pooled for each time point and anti-IgG titers against *P. aeruginosa* PAO1 were determined by ELISA methods described above. The half-lives of WVDC-0357 and WVDC-0496 were determined using a one-phase exponential decay model.

2.11 Histopathology

Mouse lungs were prepared and sent for histopathological analysis. Following challenge and euthanasia of mice in the pneumonia model, the left lobe of the lung was placed in 10% neutral buffered formalin. Paraffin-embedded lung sections with 5 μ m thickness were mounted on glass slides and stained with (i) hematoxylin and eosin to assess for acute inflammation and (ii) Gram stain to assess for presence of bacilli. Lungs were scored by a pathologist blinded to experimental groups.

2.12 Measurement of cytokine and chemokine levels

The concentration of CXCL-1, TNF- α , IL-1 β , IL-6, IL-10, and IFN- γ in the serum and lung supernatant was quantified using a Luminex multiplex assay (R&D). Procedures were performed according to manufacturer's instructions.

2.13 Antibody sequencing

The variable region of WVDC-0357 and WVDC-0496 were sequenced using methods by Meyer et al. (2019). Briefly, total RNA was extracted using Quick-RNA Microprep Kit (Zymo, #R1051). The cDNA was synthesized using the SMARTScribe Reverse Transcriptase kit (Clontech, #639537) with a custom template switch oligonucleotide and chain-specific reverse primers. Touchdown PCR was used to amplify the synthesized cDNA with a universal forward primer and nested chain-specific reverse primers. The amplified PCR reaction was run on a 1% agarose gel and DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen, #28704). The extracted DNA was Sanger sequenced using the IgG reverse primers.

2.14 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9. Survival data is presented as a Kaplan-Meier curve and analyzed by the log-rank (Mantel-Cox) test. All other data is presented as the mean $\pm$ standard deviation (SD). For

comparison between two groups, a two-tailed student's t-test was performed. For comparison between three or more groups, a one-way ANOVA with Dunnett's multiple comparison or Kruskal-Wallis with Dunn's multiple comparison was performed depending on the normality of the data set. P values less than 0.05 were considered statistically significant.

3 Results

3.1 Convalescent mice previously infected with *P. aeruginosa* PAO1 cultured on PIAAMV are protected against lethal challenge

We hypothesized that mice previously challenged with *P. aeruginosa* PAO1 cultured on PIAAMV would be protected from a lethal *P. aeruginosa* challenge. To test this hypothesis, *P. aeruginosa* PAO1 was cultured on PIAAMV (Figure 1A) and a sub-lethal dose (1.4×10^7 CFU) was administered IN to six-week old female CD-1 mice, which were allowed to convalesce for 34 d. Naïve and convalescent mice were then challenged IN with a lethal dose of *P. aeruginosa* PAO1 cultured on PIA (1.4×10^8 CFU), and were euthanized 16 h post-challenge to determine serum antibody titers and bacterial burden in the lungs and nasal cavity. Convalescent mice had significantly higher serum antibody titers, with a 2.62 log increase ($P < 0.0001$) in anti-IgG antibody titers compared to naïve mice (Figure 1B). Furthermore, bacterial burden was significantly lower in convalescent challenged mice compared to naïve mice with a 4.68 log reduction in CFUs in the lungs ($P < 0.01$) and a 3.98 log reduction in CFUs in the nasal wash ($P < 0.001$) (Figure 1C). Overall, these findings demonstrate that mice previously exposed to *P. aeruginosa* PAO1 cultured on PIAAMV were protected against a lethal *P. aeruginosa* challenge and produced significantly greater anti-*P. aeruginosa* antibody responses compared to naïve mice. Based on these results, we hypothesized that immunization with

P. aeruginosa cultured on PIAAMV would induce a robust production of anti-*P. aeruginosa* antibody-secreting cells that could be used for hybridoma generation.

3.2 Generation and characterization of monoclonal antibodies against *P. aeruginosa*

To generate mAbs against *P. aeruginosa*, CD-1 mice were immunized and boosted with live preparations of *P. aeruginosa* PAO1 grown on PIAAMV and following convalescence, hybridomas were formed by fusing spleen cells with immortalized myeloma cells. We screened a total of 508 hybridoma colonies and identified two IgG2b-kappa secreting hybridomas (WVDC-0357 and WVDC-0496) that had strong binding to intact *P. aeruginosa* PAO1 by ELISA (Supplementary Figure 1). WVDC-0357 and WVDC-0496 were subsequently purified for further characterization. Flow cytometry was performed to confirm binding of WVDC-0357 and WVDC-0496 to intact *P. aeruginosa* PAO1, which demonstrated an approximate 1-log shift in fluorescence above the isotype-matched control (Figure 2A). In addition, we showed that WVDC-0357 and WVDC-0496 had a dose-response profile of binding to *P. aeruginosa*. Binding kinetic studies were performed with flow cytometry, which demonstrated a dissociation constant (K_D) in the nanomolar range. The obtained K_D values were 14.96 ± 6.73 nM and 11.45 ± 4.32 nM, for WVDC-0357 and WVDC-0496, respectively (Figure 2B, Supplementary Table 2). Sequencing of the antibody variable region revealed that WVDC-0357 and WVDC-0496 share similar variable regions with point mutations encoded in LFR3 and LFR4. For both antibodies, the light chain sequence was derived from the murine germline sequences IGKV1-122 and IGKJ2, and the heavy chain sequence was derived from the murine germline sequences IGHV1-52, IGHV1-61, IGHD1-1, and IGHJ4.

To further characterize the binding specificity of WVDC-0357 and WVDC-0496, an ELISA screen was conducted against a panel of

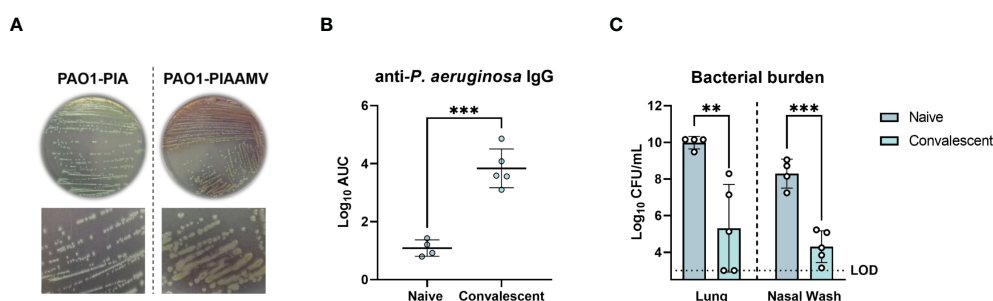


FIGURE 1

Protection of convalescent mice against lethal *P. aeruginosa* challenge after prior infection with *P. aeruginosa* PAO1 cultured on Pseudomonas isolation agar supplemented with ammonium metavanadate (PIAAMV). CD-1 mice were challenged with a sub-lethal intranasal dose (1.4×10^7 CFU) of *P. aeruginosa* PAO1 cultured on PIAAMV and allowed to convalesce before being challenged with a lethal intranasal dose of *P. aeruginosa* PAO1 cultured on Pseudomonas isolation agar (PIA). Mice were euthanized at 16 h post-challenge. (A) Comparison of colony morphology between *P. aeruginosa* PAO1 cultured on PIA and *P. aeruginosa* PAO1 cultured on PIAAMV used for dose preparation. Growth of *P. aeruginosa* PAO1 on PIAAMV induced a mucoid colony morphology. (B) Serum anti-*P. aeruginosa* IgG titers determined by ELISA are represented by area under the curve (AUC) values. Differences in serum antibody titers were determined using a two-tailed student's t-test. *** $P < 0.001$ compared to naïve mice. (C) Bacterial burden in the lung and nasal wash was determined at 16 hours post-challenge. Differences in bacterial burden were determined using a two-tailed student's t-test. ** $P < 0.01$ and *** $P < 0.001$ compared to naïve mice. LOD, limit of detection.

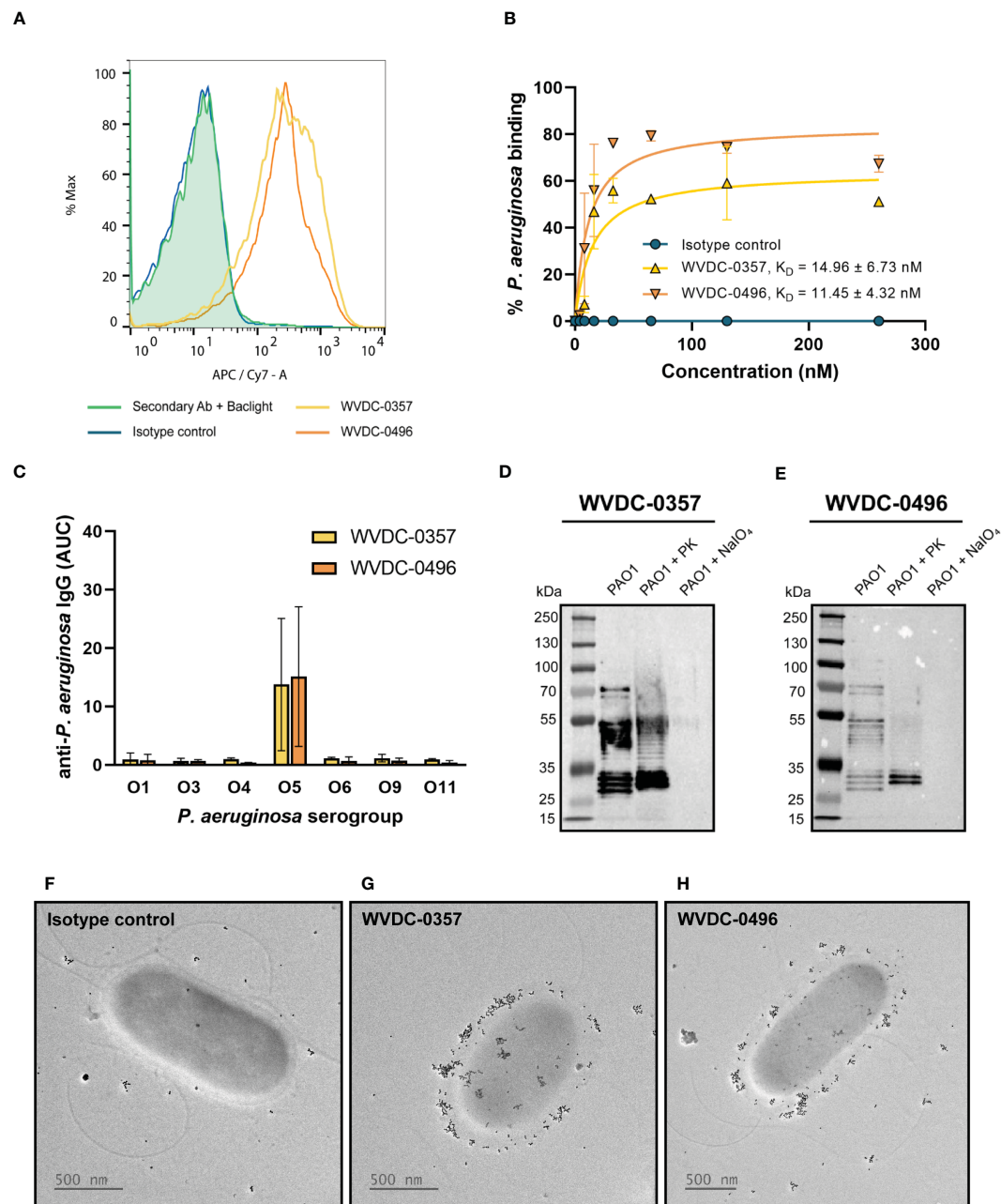


FIGURE 2

Characterization of anti-*P. aeruginosa* mAbs, WVDC-0357 and WVDC-0496. **(A)** Fluorescence-activated cell sorting (FACS) analysis of binding to *P. aeruginosa* was performed using WVDC-0357, WVDC-0496, and an isotype control mAb. **(B)** Binding kinetic analysis revealed dissociation constants (K_D) of 14.96 ± 6.73 nM and 11.45 ± 4.32 nM for WVDC-0357 and WVDC-0496, respectively, using a non-linear regression model. **(C)** ELISA was used to determine anti-*P. aeruginosa* IgG titers against different serogroups, represented as area under the curve (AUC) values. **(D, E)** Immunoblot analysis of *P. aeruginosa* outer membrane fractions treated with proteinase K or sodium periodate, and probed with WVDC-0357 and WVDC-0496. **(F-H)** Electron microscopy imaging of *P. aeruginosa* treated with an isotype control mAb, WVDC-0357, or WVDC-0496, labeled with a gold-conjugated secondary antibody. PK, proteinase K; NaIO₄, sodium periodate.

P. aeruginosa strains (Supplementary Table 1), which included clinical strains obtained from Dr. Robert Ernst (Burns et al., 2001) and the Multidrug Resistant Organism Repository and Surveillance Network (MSRN) (Lebreton et al., 2021). The ELISA results showed that these mAbs bind specifically to *P. aeruginosa* strains from serogroup O5 (Figure 2C). To investigate the influence of stress conditions on binding, ELISAs were also conducted with each serogroup O5 strain cultivated on PIAAMV. There was a significant increase in the binding

of WVDC-0357 and WVDC-0496 to serogroup O5 strains when cultivated on PIAAMV compared to PIA (Supplementary Figure 2). These findings suggest that WVDC-0357 and WVDC-0496 not only recognize serogroup O5 strains under standard growth conditions, but also demonstrate increased binding when these strains are exposed to stress induced by ammonium metavanadate.

Given the high specificity of WVDC-0357 and WVDC-0496 for serogroup O5 strains, we hypothesized that they targeted the

polysaccharide component of *P. aeruginosa*. To test this hypothesis, a western blot was performed with *P. aeruginosa* PAO1 outer membrane fractions treated with proteinase K, to digest proteins, or sodium periodate, to degrade polysaccharides. The results of the western blot analysis indicated that WVDC-0357 and WVDC-0496 recognized polysaccharide as the antigen, as evidenced by the banding pattern observed (Figures 2D, E, Supplementary Figure 3). Furthermore, treatment of *P. aeruginosa* PAO1 with proteinase K did not alter binding, suggesting that the antibodies do not bind proteins; however, treatment of *P. aeruginosa* PAO1 with sodium periodate completely ablated binding, suggesting that these antibodies specifically bind polysaccharides (Figures 2D, E). In addition, we performed western blotting on a *P. aeruginosa* PAO1 transposon mutant deficient in the gene *wbpC*, which is involved in O-antigen biosynthesis and functions as a possible O-acetyltransferase (King et al., 2009). WVDC-0357 and WVDC-0496 did not bind to the transposon mutant for the gene *wbpC* (Supplementary Figure 4), further confirming that these antibodies target the O-antigen of LPS. Additional experiments were conducted against unrelated antigens, such as dextran, fetuin, and gelatin, however WVDC-0357 and WVDC-0496 did not show binding to these antigens (Supplementary Figure 5).

Lastly, we performed immunogold-labeling of *P. aeruginosa* to visualize surface binding by WVDC-0357 and WVDC-0496. Bacteria incubated with the isotype control mAb displayed no detectable surface binding of antibody (Figure 2F). In contrast, bacteria incubated with WVDC-0357 and WVDC-0496

demonstrated diffuse binding of antibody along the entire envelope surface (Figures 2G, H).

3.3 WVDC-0357 and WVDC-0496 induce bacterial aggregation

Antibodies can play a critical role in the host defense against microbial pathogens through their canonical functions, such as aggregation and neutralization, complement binding, and opsonophagocytosis (Dimitrov and Lacroix-Desmazes, 2020). To investigate the functional properties of WVDC-0357 and WVDC-0496 in inducing aggregation of *P. aeruginosa*, bacterial growth curves were performed in UV-transparent cuvettes, whereby *P. aeruginosa* PAO1 was cultured in lysogeny broth with the inclusion of an isotype control mAb, WVDC-0357, or WVDC-0496. Cultures were incubated statically at 37°C and the absorbance at 600 nm was measured over a 24 h period. We observed a significant decrease in absorbance values in cultures containing WVDC-0357 and WVDC-0496 compared to the isotype control mAb, with significant differences ($P < 0.0001$) at all time points starting at 2 h (Figure 3A). Furthermore, visualization of the cuvettes demonstrated clear differences in the turbidity of growth cultures at 24 h (Figure 3B). To determine if the decrease in turbidity was due to bacterial aggregation and precipitation, *P. aeruginosa* PAO1 was stained with BacLight red bacterial stain, treated with an isotype control mAb, WVDC-0357, or WVDC-0496, and visualized with fluorescence microscopy for bacterial aggregates. Visualization of

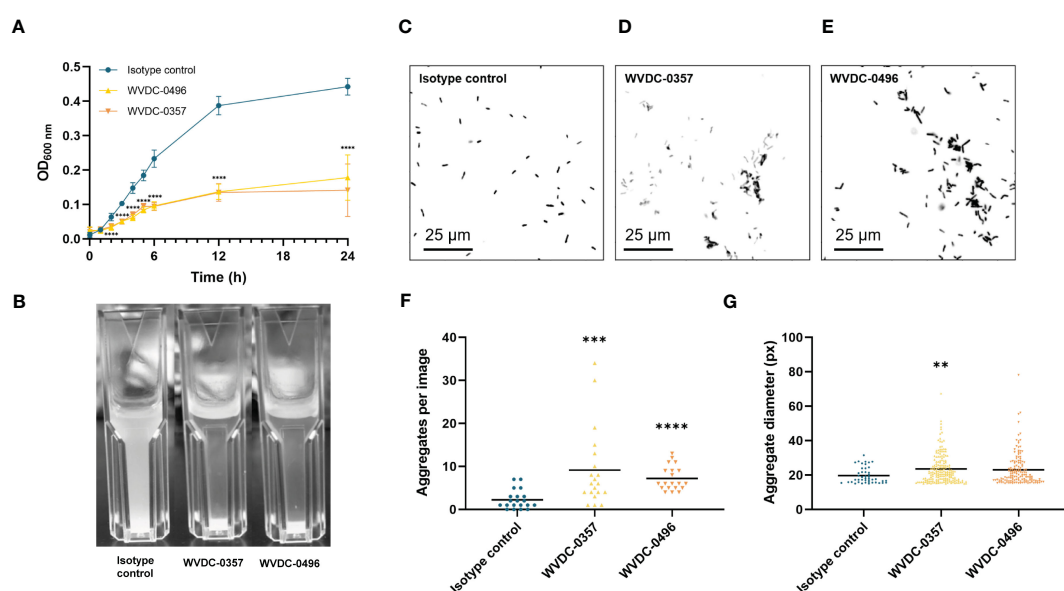


FIGURE 3

WVDC-0357 and WVDC-0496 promote bacterial agglutination. (A) In UV-transparent cuvettes, fresh cultures of *P. aeruginosa* PAO1 in lysogeny broth were incubated with an isotype control mAb, WVDC-0357, or WVDC-0496 at 100 µg/ml. Cuvettes were incubated statically at 37°C and the absorbance of growth cultures at 600 nm were measured over the course of 24 h. Differences in absorbance were analyzed using a one-way ANOVA with Dunnett's multiple comparison test. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ compared to the isotype control. (B) After incubation for 24 h, cuvettes were photographed for visual inspection of turbidity. (C-E) Representative fluorescent microscopy images of *P. aeruginosa* PAO1 stained with BacLight Red and treated with an isotype control mAb, WVDC-0357, or WVDC-0496. (F, G) Random area images were analyzed with an aggregation cluster pipeline in CellProfiler for number of aggregates per image and aggregate diameter. Differences in aggregates were calculated using the Kruskal Wallis test with Dunn's multiple comparison. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to the isotype control.

fluorescent *P. aeruginosa* PAO1 treated with WVDC-0357 and WVDC-0496 demonstrated an increase in bacterial aggregates compared to the isotype control (Figures 3C-E). To quantify the number and size of bacterial aggregates, randomly imaged areas were analyzed for number of aggregates and mean aggregate diameter using CellProfiler. For WVDC-0357 and WVDC-0496, there were significant increases in the total number of aggregates per image compared to the isotype control (Figure 3F). Furthermore, the mean aggregate diameter was greater for WVDC-0357 and WVDC-0496 compared to the isotype control (Figure 3G). Additional experiments were performed to determine the role of WVDC-0357 and WVDC-0496 in mediating opsonophagocytosis and complement-mediated killing. However, data from these experiments suggest that these antibodies do not induce macrophage opsonophagocytosis or complement-mediated killing of *P. aeruginosa* under tested conditions (Supplementary Figure 6). Collectively, these results demonstrate that WVDC-0357 and WVDC-0496 induce bacterial aggregation but do not promote opsonophagocytic or complement-mediated killing of *P. aeruginosa*.

3.4 WVDC-0357 and WVDC-0496 mediate direct antibacterial activity against *P. aeruginosa*

Antibodies display a wide and diverse set of functions, yet recent studies have demonstrated the importance of the non-canonical function of antibodies in host immunity (Dimitrov and Lacroix-Desmazes, 2020). Besides the effector system pathways, antibodies are able to mediate antimicrobial activity through mechanisms such

as direct pathogen inactivation (Dimitrov and Lacroix-Desmazes, 2020; Hotinger and May, 2020). To investigate the antibacterial activity of WVDC-0357 and WVDC-0496, bacterial killing assays were performed, whereby mid-log phase *P. aeruginosa* PAO1 was incubated with either an isotype control mAb, WVDC-0357, or WVDC-0496. After vortexing to ensure dissociation of any aggregates, the bacteria were plated for CFU enumeration. Results showed that WVDC-0357 and WVDC-0496 exhibited a dose-dependent killing effect on *P. aeruginosa* PAO1 with significant reductions in viable bacteria when compared to isotype control mAb-treated bacteria (Figure 4A). In addition, we evaluated the antibacterial activity of WVDC-0357 and WVDC-0496 against a diverse set of *P. aeruginosa* serogroup O5 strains, including strains sourced from the MRSN collection. This collection encompasses a wide range of *P. aeruginosa* strains, including extensively drug-resistant (XDR) strains (Lebreton et al., 2021). The efficacy of WVDC-0357 and WVDC-0496 varied among strains, yet collectively there was a significant increase in the percentage of *P. aeruginosa* killing compared to the isotype control (Figure 4B). Overall, these findings indicate that WVDC-0357 and WVDC-0496 effectively mediate direct bacterial killing of *P. aeruginosa*.

3.5 Pharmacokinetics of WVDC-0357 and WVDC-0496

Determining the pharmacokinetic profiles of WVDC-0357 and WVDC-0496 is important to ensure adequate levels of these mAbs in circulation, allowing them to exert their target function. To assess

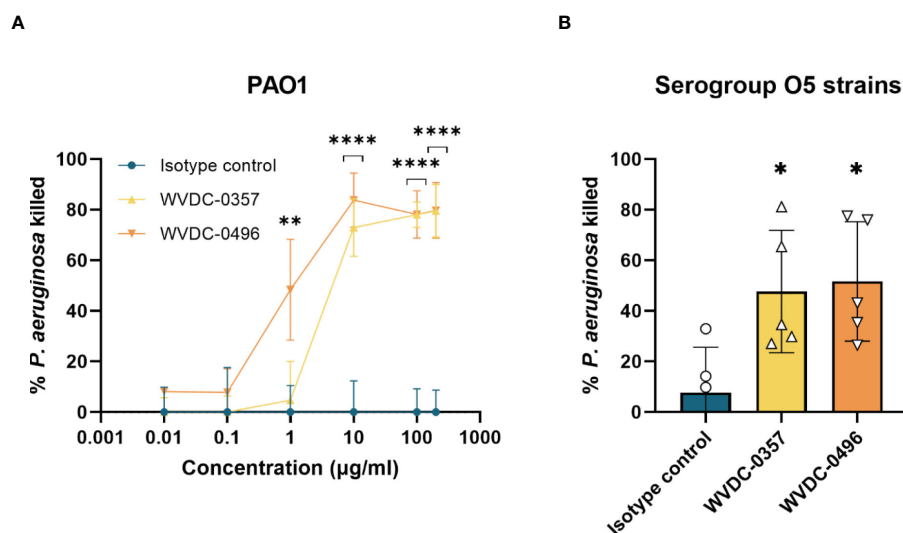


FIGURE 4

Antibacterial activity of WVDC-0357 and WVDC-0496. (A) Antibacterial activity against *P. aeruginosa* PAO1. Mid-log phase growth cultures of *P. aeruginosa* PAO1 were treated with varying concentrations of an isotype control mAb, WVDC-0357 or WVDC-0496 before plating onto Pseudomonas isolation agar (PIA) plates for CFU enumeration. The percent killing was determined by normalizing the number of CFUs from each sample to the mean CFUs of the no-antibody control. Differences in CFUs were calculated using a one-way ANOVA with Dunnett's multiple comparison test. ** $P < 0.01$. **** $P < 0.0001$ compared to the isotype control. (B) Antibacterial activity against O5 serogroup strains. *P. aeruginosa* strains PAO1, MRSN 1601, MRSN 2101, MRSN 12368, and MRSN 14981 was treated with 100 µg/mL of an isotype control mAb, WVDC-0357, and WVDC-0496, and then plated on PIA for CFU enumeration. Each data point represents the average percent killing for a specific strain. The percent killing was determined by normalizing the number of CFUs from each sample to the mean CFUs of the no-antibody control. Differences in CFUs were calculated using a one-way ANOVA with Dunnett's multiple comparison test. * $P < 0.05$ compared to the isotype control.

the pharmacokinetics of WVDC-0357 and WVDC-0496, CD-1 mice were IP administered 45 mg/kg of antibody and were submandibular bled up to 21 d post-administration to collect serum. Antibody titers were determined using ELISA against intact *P. aeruginosa* PAO1 and plotted against time to determine their half-lives using a one-phase exponential decay model. Both WVDC-0357 and WVDC-0496 had similar pharmacokinetic profiles, with half-lives of 7.094 and 7.619 d, respectively (Figure 5). These half-life values are consistent with previous reports on the half-life of murine IgG2b antibodies (Vieira and Rajewsky, 1988). These findings indicate that WVDC-0357 and WVDC-0496 are stable in circulation for up to 72 h, providing insight for subsequent *in vivo* studies on the prophylactic administration of these mAbs.

3.6 WVDC-0357 and WVDC-0496 protect mice against lethal *P. aeruginosa* bloodstream challenge

Primary bloodstream infections are one of the most lethal complications of *P. aeruginosa* infections (Wisplinghoff et al., 2004). Given the functional profile of WVDC-0357 and WVDC-0496 against *P. aeruginosa*, we evaluated the efficacy of these mAbs in a lethal sepsis model. In this model, CD-1 mice were IP administered either an isotype control mAb, *P. aeruginosa* whole cell vaccinated (*Pa* WCV) serum as a positive control (Sen-Kilic et al., 2021; Blackwood et al., 2022), WVDC-0357, or WVDC-0496,

and challenged 12 h later with a lethal IP dose of *P. aeruginosa* PAO1 (5×10^5 CFU) (Figure 6A). Survival was monitored over the course of 96 h, and the severity of disease was assessed by scoring the mice daily based on clinical outcomes such as appearance, activity, eye closure, respiration quality, body temperature, and body weight. Each variable was given a score ranging from 0 to 4, and mice that had a total cumulative score of 12 or a score of 4 in any individual category were humanely euthanized. During the 96 h observation period, mice in the isotype control group quickly showed an increase in clinical scores, with a majority requiring euthanasia between 6 and 12 h post-challenge (Figure 6B, Supplementary Figure 7). As a secondary metric of overall health, temperature was a key indicator of morbidity. Mice that did not survive had on average a rectal temperature of 31.5°C, which was a 15% decrease from baseline. In contrast, mice administered *Pa* WCV serum, WVDC-0357, or WVDC-0496 maintained baseline rectal temperatures throughout the whole observation period (Figure 6C, Supplementary Figure 7). When looking at survival of mice, the majority of mice treated with the isotype control mAb required euthanasia within the first 24 h, and there was only a 10% survival rate at the conclusion of the observation period (Figure 6D). In contrast, mice treated with *Pa* WCV serum, 45 mg/kg of WVDC-0357, or 45 mg/kg of WVDC-0496 were fully protected and had a 100% survival rate (Figure 6D). This study was repeated with the addition of a 15 mg/kg dosing group for both WVDC-0357 and WVDC-0496, and these mice still demonstrated a 100% survival rate (Figure 6D).

To quantify the level of bacterial burden and inflammatory cytokines in the early stages of infection, an additional set of mice were euthanized at 6 h post-challenge. WVDC-0357 and WVDC-0496 significantly reduced bacterial burden to the limit of detection in the blood, kidney, and spleen, with a reduction in CFUs similar to the positive control *Pa* WCV serum group (Figure 6E). Compared to the isotype control mAb group, protected mice had a 2.11, 1.38, and 1.44 fold log reduction in CFUs in the blood, kidney, and spleen, respectively. The level of inflammatory cytokines and chemokines was drastically reduced in mice treated with WVDC-0357 and WVDC-0496 compared to isotype control mice (Figures 7A-E). The concentration of cytokines and chemokines was similar between the positive control *Pa* WCV serum group and WVDC-0357 and WVDC-0496, which was near the lower limit of detection. WVDC-0357 and WVDC-0496 significantly reduced the levels of CXCL-1, IFN- γ , TNF- α , IL-1 β and IL-6 compared to the isotype control mAb (Figures 7A-E).

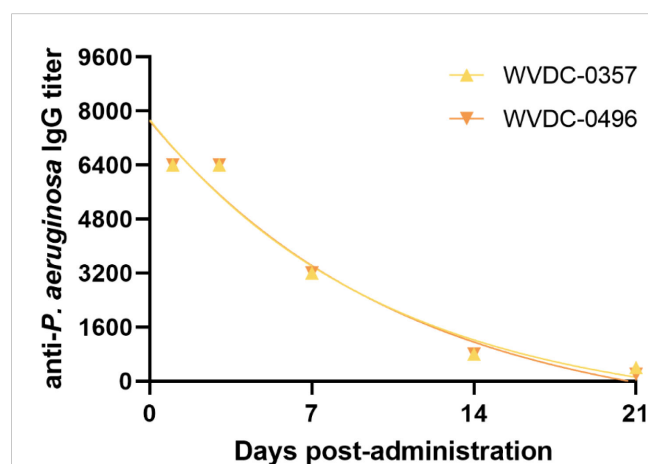


FIGURE 5
Pharmacokinetics of WVDC-0357 and WVDC-0496. CD-1 mice were intraperitoneally (IP) administered 45 mg/kg of WVDC-0357 or WVDC-0496 and submandibular bled at various time points over the course of 21 d to assess serum IgG titers against *P. aeruginosa* PAO1 using ELISA. The half-life ($t_{1/2}$) of WVDC-0357 and WVDC-0496 was determined using a one-phase exponential decay model.

3.7 WVDC-0357 and WVDC-0496 protect mice against acute *P. aeruginosa* lung challenge

We next evaluated the efficacy of WVDC-0357 and WVDC-0496 in an acute pneumonia model. CD-1 mice were IP administered either an isotype control mAb, *Pa* WCV serum, WVDC-0357, or WVDC-0496. After 12 h, mice were IN challenged with *P. aeruginosa* PAO1 (2×10^7 CFU) and euthanized at 16 h post-challenge to assess for correlates of protection

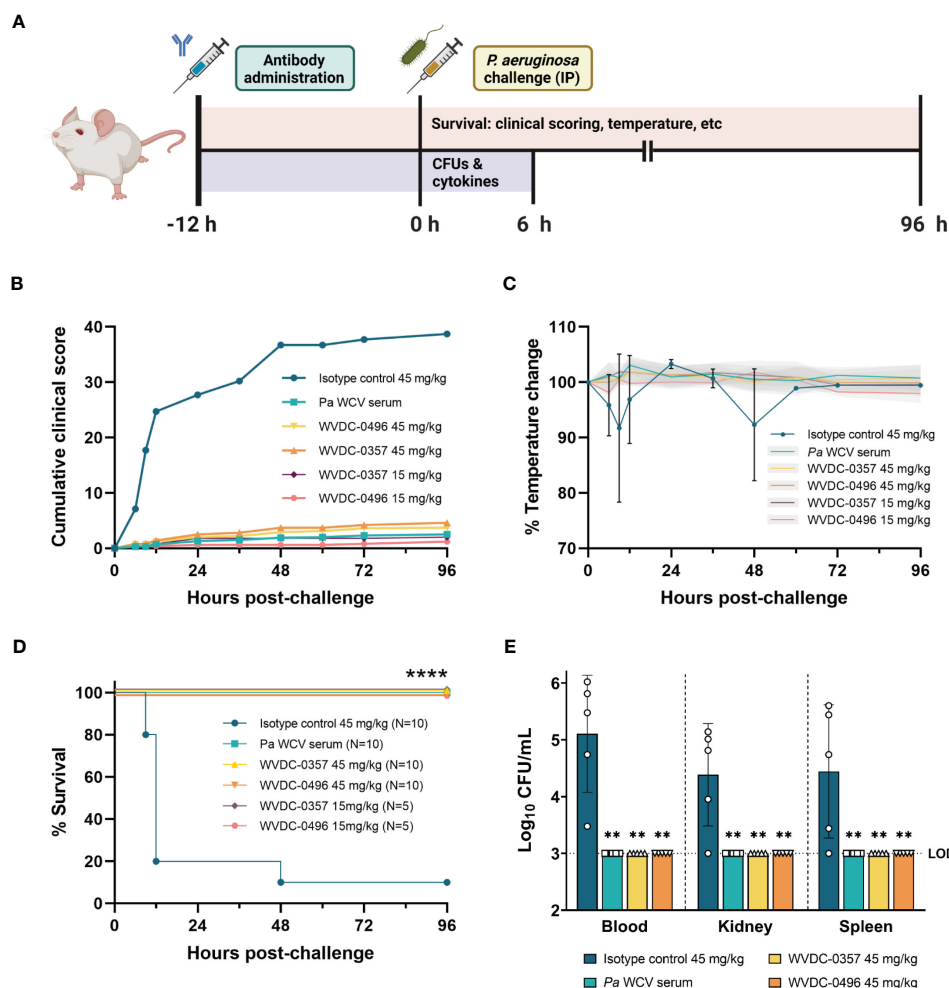


FIGURE 6

WVDC-0357 and WVDC-0496 protect mice following lethal bloodstream challenge with *P. aeruginosa*. (A) CD-1 mice were prophylactically administered either an isotype control mAb, *P. aeruginosa* whole cell vaccine (*Pa* WCV) serum, WVDC-0357, or WVDC-0496. After 12 h, mice were challenged with an intraperitoneal (IP) dose of *P. aeruginosa* PAO1 (5×10^5 CFU) and survival was monitored for 96 h. A separate set of mice was euthanized at 6 h post-challenge to determine bacterial burden in the blood, kidney, and spleen. (B) In the survival model, clinical scores were calculated at the indicated time points. Shown is the cumulative total of average clinical scores for each group. (C) Temperature was a key indicator of morbidity in mice. Shown is the average percent temperature change of each group, with the standard deviation of the isotype control mice shown in black error bars and the standard deviation of all other groups shown as gray shaded regions. (D) The survival of mice is represented as Kaplan-Meier curves, with 5–10 mice in each group. Differences in survival were calculated using the log-rank test. **** $P < 0.0001$ compared to isotype control. (E) At 6 h post-challenge, the blood, kidney, and spleen homogenate were serially diluted and plated to determine bacterial burden. Differences in CFUs were calculated using the Kruskal Wallis test with Dunn's multiple comparison. ** $P < 0.01$ compared to the isotype control. Abbreviations: LOD, limit of detection.

(Figure 8A). Our results showed that WVDC-0357 and WVDC-0496 significantly reduced bacterial burden in the respiratory tract of the mice. Compared to mice treated with an isotype control mAb, mice treated with WVDC-0357 had a 2.16 log reduction in lung CFUs ($P < 0.001$) and 2.46 log reduction in nasal wash CFUs ($P < 0.0001$), while mice treated with WVDC-0496 had a 2.71 log reduction in lung CFUs ($P < 0.0001$) and a 2.35 log reduction in nasal wash CFUs ($P < 0.0001$) (Figure 8B). This reduction in bacterial burden was similar to that observed in mice treated with *Pa* WCV serum. Wet lung weight was used as a marker of lung edema and we found a significant decrease in the lung weight of mice treated with *Pa* WCV serum, WVDC-0357, and WVDC-0496 when compared to the isotype control (Figure 8C). Additionally, the temperature of the mice was recorded as an overall metric of health. The average

temperature in mice treated with *Pa* WCV serum, WVDC-0357, or WVDC-0496 was close to the physiological temperatures of non-challenged mice, with only a 2–3% decrease (Figure 8D). In contrast, mice treated with the isotype control mAb had an average decrease in rectal temperature of 13% when compared to the physiological temperature of non-challenged mice (Figure 8D).

3.8 WVDC-0357 and WVDC-0496 reduce lung inflammation post-challenge

Acute lung infections caused by *P. aeruginosa* can trigger strong inflammatory cytokine and chemokine responses that are often associated with deleterious lung damage (Yanagihara et al., 2000;

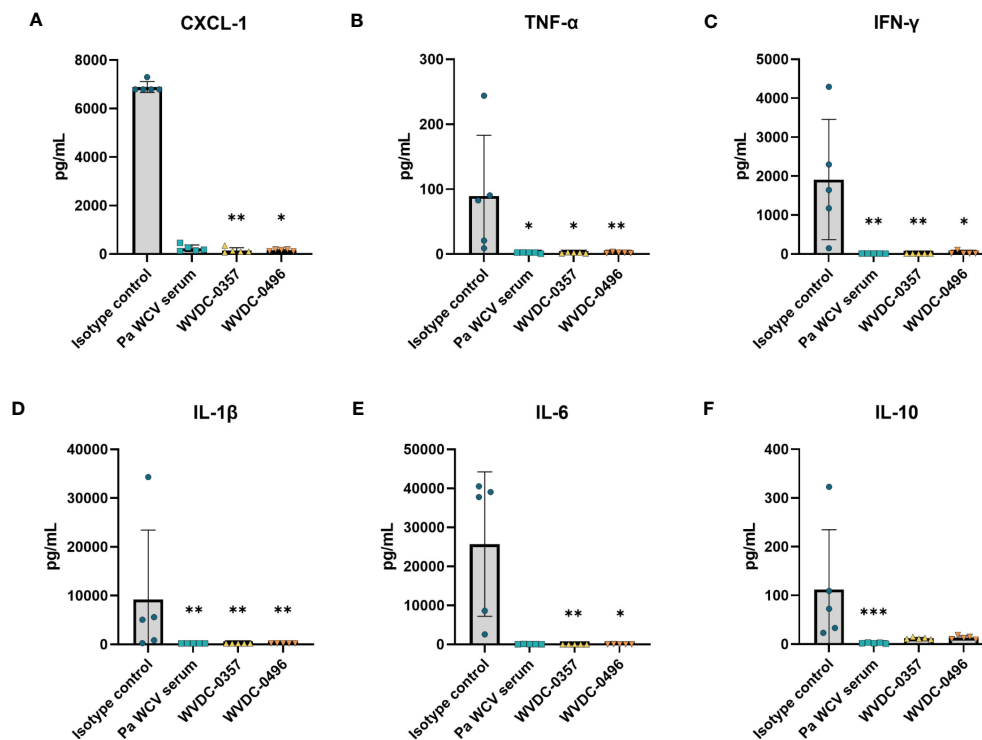


FIGURE 7

Level of inflammatory cytokines and chemokines in serum following lethal bloodstream challenge with *P. aeruginosa*. CD-1 mice were prophylactically administered an isotype control mAb, *P. aeruginosa* whole cell vaccine (Pa WCV) serum, WVDC-0357, or WVDC-0496, and challenged 12 h later with an intraperitoneal (IP) dose of *P. aeruginosa* PAO1 (5×10^5 CFU). Mice were euthanized at 6 h post-challenge, and the serum concentration of (A) CXCL-1, (B) IFN-γ, (C) TNF-α, (D) IL-1β, (E) IL-6, and (F) IL-10 was measured with the Luminex assay. Differences in cytokines and chemokines were calculated using the Kruskal Wallis test with Dunn's multiple comparison. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the isotype control.

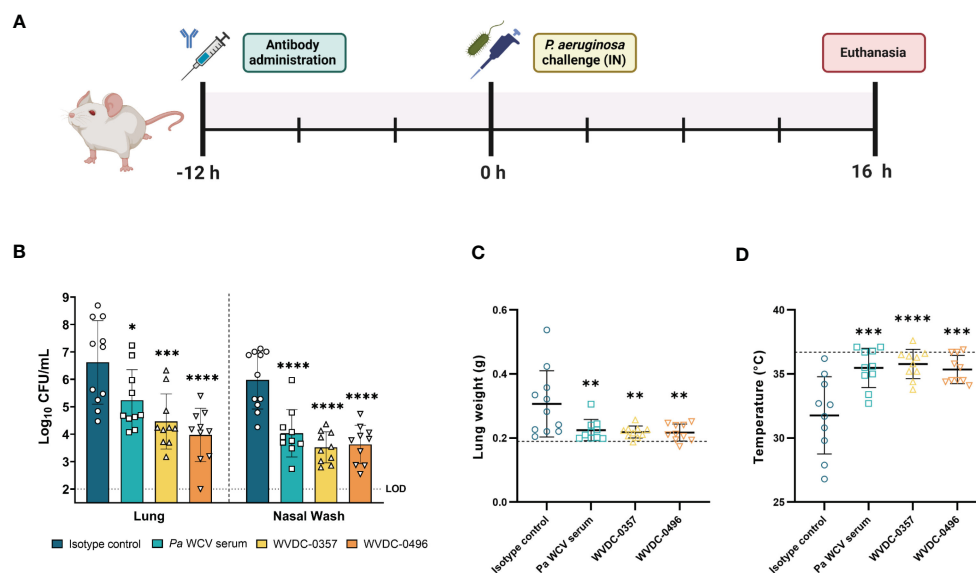


FIGURE 8

WVDC-0357 and WVDC-0496 enhance bacterial clearance in an acute pneumonia model. (A) CD-1 mice were prophylactically administered either an isotype control mAb, *P. aeruginosa* whole cell vaccine (Pa WCV) serum, WVDC-0357, or WVDC-0496, and after 12 h were challenged with an intranasal (IN) dose of *P. aeruginosa* PAO1 (2×10^7 CFU). Mice were euthanized at 16 h post-challenge. (B) Bacterial burden in the lung and nasal wash of mice. Differences in CFUs were calculated using a one-way ANOVA with Dunnett's multiple comparison test. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ compared to the isotype control. (C) Lung weights of mice were recorded at euthanasia. The dashed line represents the lung weight of naive, non-challenged mice. Differences in lung weight were calculated using a one-way ANOVA with Dunnett's multiple comparison test. ** $P < 0.01$ compared to the isotype control. (D) Rectal temperatures of mice at were recorded at euthanasia. The dashed line represents the rectal temperature of naive, non-challenged mice. Differences in temperature were calculated using a one-way ANOVA with Dunnett's multiple comparison test. *** $P < 0.001$, **** $P < 0.0001$ compared to the isotype control. Abbreviations: LOD, limit of detection.

Mijares et al., 2011; Lin and Kazmierczak, 2017). We hypothesized that prophylactic treatment of mice with WVDC-0357 and WVDC-0496 would decrease key inflammatory markers and decrease total inflammation in the lung following intranasal challenge with *P. aeruginosa*. We found that mice treated with WVDC-0357 and WVDC-0496 had significantly lower levels of CXCL-1, TNF- α , IL-1 β and IL-6, and IL-10 compared to mice treated with an isotype control mAb (Figure 9). Histopathological examination of the lungs demonstrated clear differences in the area and pattern of inflammation. In the absence of challenge, negative control mice displayed no signs of inflammation (Figure 10A). In contrast, mice in the isotype control group displayed a diffuse pattern of alveolar inflammation, characterized predominantly by neutrophils (Figure 10B). Gram staining revealed the presence of live bacilli with a diffuse distribution in all samples (Figure 10B). Among mice treated with *Pa* WCV serum, WVDC-0357, or WVDC-0496, there was a significant reduction in the mean area of inflammation (Figures 10C–F). Furthermore, inflammation was largely restricted to the peribronchial zones, migration through the pulmonary parenchyma was limited, and Gram staining revealed the absence of bacilli (Figures 10C–E, G). Taken together, these findings suggest that WVDC-0357 and WVDC-0496 are effective in mitigating key inflammatory markers and restricting the spread of inflammation in the lung against *P. aeruginosa* challenge.

4 Discussion

P. aeruginosa is one of the most commonly encountered nosocomial pathogens and is responsible for complications such as VAP and primary bloodstream infections (Spagnolo et al., 2021). Considering the prevalence of MDR strains of *P. aeruginosa* and mortality associated with these infections, there is an urgent need to develop alternative therapeutics against *P. aeruginosa*. In this study, we generated WVDC-0357 and WVDC-0496, which are highly specific to the O-antigen LPS of *P. aeruginosa*. Furthermore, we demonstrated that prophylactic administration of WVDC-0357 and WVDC-0496 is highly effective at protecting mice against lethal bacteremia and pneumonia.

We had previously demonstrated that when *P. aeruginosa* PAO1 is cultured on PIAAMV, a host of bacterial genes are differentially regulated when compared to PIA alone, including an upregulation in genes for LPS synthesis (Damron et al., 2013). Furthermore, convalescent mice previously infected with *P. aeruginosa* PAO1 cultured on PIAAMV were protected against a lethal *P. aeruginosa* challenge (Figures 1B, C). We hypothesized that the predominant antibody response triggered by *P. aeruginosa* PAO1 cultured on PIAAMV was primarily against outer polysaccharides and used convalescent mice who had been previously infected with *P. aeruginosa* PAO1 cultured on

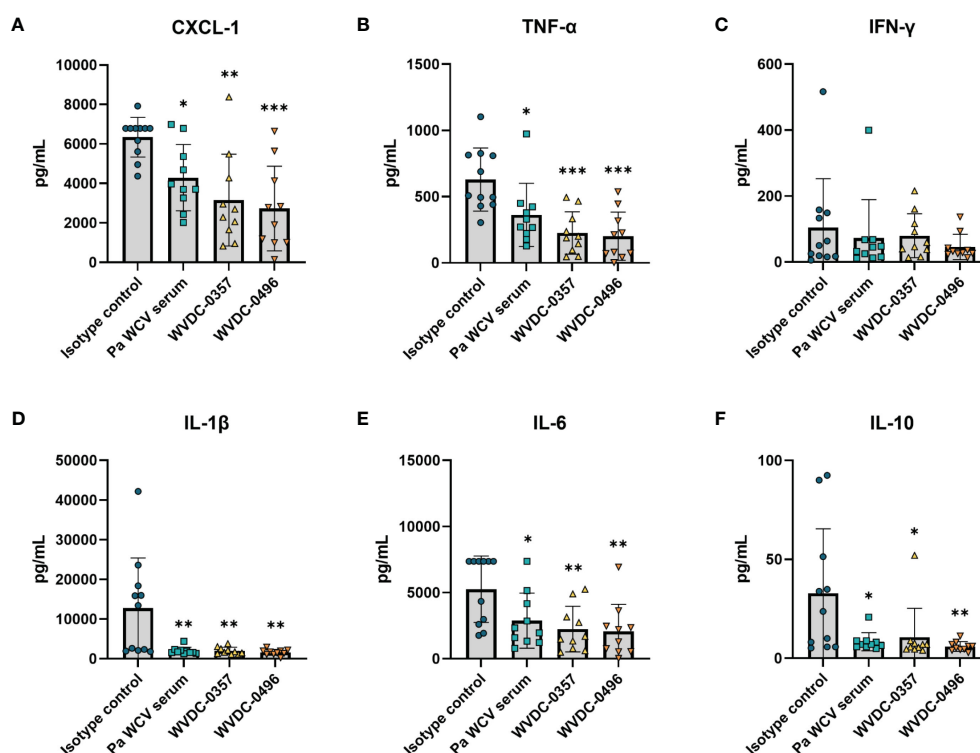


FIGURE 9

Level of inflammatory cytokines and chemokines in the lung following acute pneumonia challenge. CD-1 mice were prophylactically administered either an isotype control mAb, *Pa* WCV serum, WVDC-0357, or WVDC-0496, and challenged 12 h later with an intranasal dose of *P. aeruginosa* PAO1 (2×10^7 CFU). Mice were euthanized 16 h post-challenge and the concentration of (A) CXCL-1, (B) IFN- γ , (C) TNF- α , (D) IL-1 β , (E) IL-6, and (F) IL-10 in the lung supernatant was measured using the Luminex assay. Differences in cytokines and chemokines were calculated using a one-way ANOVA with Dunnett's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to the isotype control.

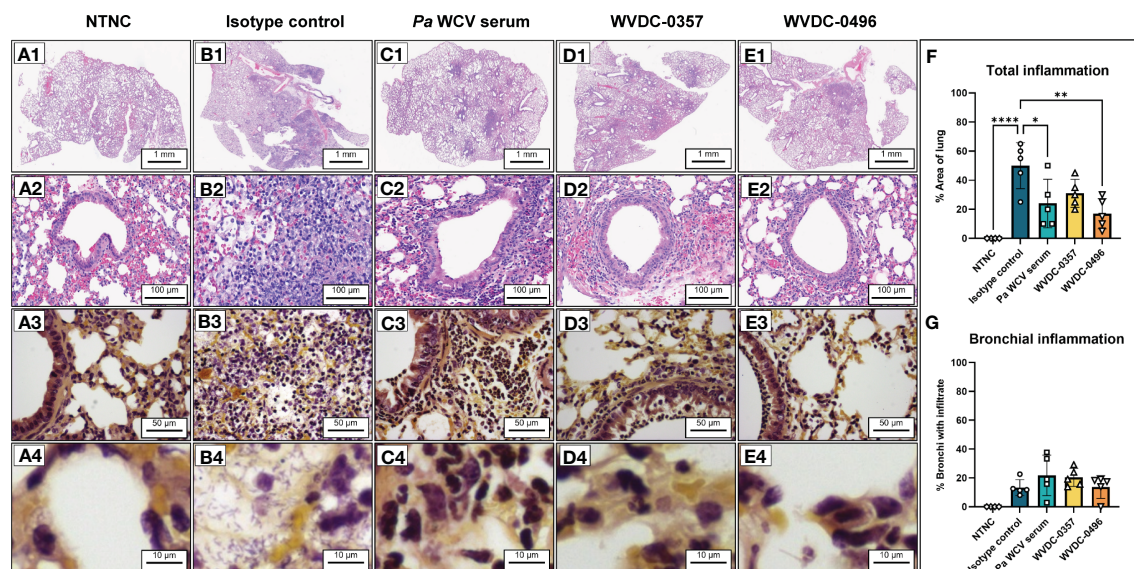


FIGURE 10

Representative hematoxylin and eosin (H&E) and Gram-stained sections obtained from mouse lungs following acute pneumonia challenge. (A) Lung from non-treated, non-challenged (NTNC) mouse showing (A1) unremarkable pulmonary parenchyma with no inflammation (A2) normal lung architecture with no alveolar or peribronchial acute inflammation, and (A3, A4) Gram stain revealing an absence of bacteria and inflammatory cells in the alveoli, bronchial lumen and peribronchial areas. (B) Lung from isotype control mAb-treated mouse displaying (B1) diffuse acute inflammation, (B2) acute inflammation of alveoli characterized by loose sheets of neutrophils, and (B3, B4) Gram stain revealing aggregates of bacilli in the alveoli and interstitium. (C) Lung from *P. aeruginosa* whole cell vaccine (Pa WCV) serum-treated mouse showing (C1) decreased net inflammation with inflammation largely restricted to peribronchial zones, (C2) acute inflammation surrounding bronchioles with relative preservation of bronchioles, and (C3, C4) Gram stain revealing an absence of bacilli in alveoli, interstitium and bronchi with surrounding peribronchial neutrophils. (D) Lung from WVDC-0357 treated mouse displaying (D1) patchy peribronchial inflammation, (D2) peribronchial acute inflammation similar to Pa WCV serum mice, and (D3, D4) Gram stain revealing an absence of bacilli in alveoli and bronchus and a layer of peribronchial neutrophils. (E) Lung from WVDC-0496 treated mouse demonstrating (E1) reduced acute inflammation restricted to rare peribronchial zones, (E2) peribronchial acute inflammation with rare, minor intraluminal infiltrate, and (E3, E4) Gram stain revealing an absence of bacilli and some neutrophils present along bronchial wall. (F) Mean lung area affected by inflammation across all groups. Differences in mean area of inflammation were calculated using a one-way ANOVA with Dunnett's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ compared to the isotype control. (G) Mean proportion of bronchial lumina affected by inflammation across all groups.

PIAAMV to raise therapeutic antibodies against *P. aeruginosa*. We utilized splenocytes derived from convalescent mice previously infected with *P. aeruginosa* PAO1 cultured on PIAAMV for hybridoma generation and identified two IgG2b mAbs, WVDC-0357 and WVDC-0496, that consistently screened positive against intact *P. aeruginosa* PAO1. Furthermore, sequencing of the variable regions of WVDC-0357 and WVDC-0496 revealed that these mAbs share the same germline genes, suggesting clonal selection of this particular epitope.

Through comprehensive ELISA and western blotting analysis, we demonstrated the specific binding of WVDC-0357 and WVDC-0496 to the O-antigen LPS of *P. aeruginosa* belonging to serogroup O5 (Figures 2C, D). LPS is a major component of the outer membrane and key virulence factor of *P. aeruginosa*, capable of eliciting inflammatory responses and inhibiting host-mediated bacterial defenses. The heterogeneity in the structure of the O-antigen component of LPS is the primary basis for serotyping schemes, which have evolved from traditional serological methods based on the International Antigenic Typing Scheme (IATS) (Liu et al., 1983; Liu and Wang, 1990) to next-generation sequencing-based approaches (Thrane et al., 2016). Based on the O-antigen gene cluster, *P. aeruginosa* can be classified into 12 serogroups, and several PCR-based methods have been developed for rapid serogroup identification (Li et al., 2018; Nasrin et al., 2022; Wang

et al., 2022). Rapid identification of *P. aeruginosa* by its serogroup not only allows for targeted treatment approaches, but also mitigates the risk of antibiotic resistance associated with broad-spectrum antibiotics. Our findings highlight the potential therapeutic effect of WVDC-0357 and WVDC-0496 towards serogroup O5 strains, even demonstrating efficacy against antibiotic-resistant *P. aeruginosa* strains. Interestingly, WVDC-0357 and WVDC-0496 exhibited enhanced binding against serogroup O5 strains cultivated in stressed growth conditions induced by ammonium metavanadate. This highlights the potential of these antibodies to function in various environmental contexts, ranging from acute infection settings to chronic infection settings.

Based on the known prevalence of serogroup O5 strains, WVDC-0357 and WVDC-0496 could be utilized for the treatment or prevention of 5-10% of *P. aeruginosa* infections (Thrane et al., 2016; Freschi et al., 2019). The narrow binding spectrum of these antibodies is beneficial as it suggests that they could be administered with less off-target effects on the natural microbial populations. However, considering the need for a comprehensive therapeutic approach, we propose that creating combinations of antibodies that target most of the clinically relevant LPS serogroups of *P. aeruginosa* is a viable solution for expanding their application.

In this study, we demonstrated the efficacy of WVDC-0357 and WVDC-0496 in mitigating *P. aeruginosa* across multiple models of infection. When untreated mice are challenged with a lethal dose of *P. aeruginosa*, they typically become morbid and require humane euthanasia within 24 to 48 h, with pathogenicity driven in large part by endotoxic shock (Secher et al., 2011; DiGiandomenico et al., 2012). In our mouse models of infection, we demonstrated that prophylactic administration of WVDC-0357 and WVDC-0496 was able to improve survival of mice and significantly reduce bacterial burden (Figures 6 and 8). Limiting the spread of infection is invariably important in preventing the onset of lethal endotoxic shock. Dysregulation of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) and anti-inflammatory cytokines (IL-10) are often associated with life-threatening infection or presence of endotoxin. The chemokine CXCL-1 is associated with neutrophil recruitment and is important in combating early infection, although prolonged neutrophil accumulation is associated with deleterious inflammation (Sun et al., 2009). In our sepsis and pneumonia models, the cytokine profile suggested that WVDC-0357 and WVDC-0496 were able to attenuate severe infection and endotoxic shock from taking place (Figures 7, 9). While the findings from our study provide valuable insights into the prophylactic use of WVDC-0357 and WVDC-0496, future studies will involve evaluating the efficacy of these mAbs in a therapeutic model to fully assess their potential as a treatment option for *P. aeruginosa* infections.

Our study aligns with previous research efforts that have developed anti-*P. aeruginosa* mAbs targeting the O-antigen of LPS. These studies demonstrated efficacy in terms of bacterial clearance and protection against infection in mouse models, however these mAbs were specifically directed against IATs serotypes O6 or O11 (Akiyama et al., 2000; Hemachandra et al., 2001; Horn et al., 2010; Xie et al., 2010; Que et al., 2014). In order to address the diverse strains of *P. aeruginosa* encountered in clinical settings, it becomes important to expand the armamentarium of antibodies to include a wider array of serotypes, such as O5. The development of WVDC-0357 and WVDC-0496 represents a significant contribution to the overall effort of providing a more comprehensive and effective antibody-based therapy against *P. aeruginosa* infections. In addition to the specificity for the O-antigen of LPS, our study revealed a unique aspect of these antibodies. Despite Fc effector functions being typically considered essential for antibacterial mAbs, WVDC-0357 and WVDC-0496 demonstrated direct bactericidal activity. While it has been reported that directly bactericidal antibodies usually target essential outer membrane proteins (Šadžiene et al., 1993; Šadžiene et al., 1994; Goel and Kapil, 2001; Storek et al., 2018), the identification of bactericidal antibodies that specifically target polysaccharides is less frequently reported. However, in a study by Yano et al., they identified that their directly bactericidal pneumococcal capsular polysaccharide mAbs against *Streptococcus pneumoniae* altered the quorum sensing response, which led to a modified bacterial agglutination and ultimately fratricide (Yano et al., 2011). We speculate that the binding of WVDC-0357 and WVDC-0496 to *P. aeruginosa* may similarly alter

the quorum sensing response, leading to bacterial agglutination and fratricide. Additionally, the structure of LPS is influenced by its local ionic environment, with divalent cations stabilizing LPS in the outer membrane while monovalent cations destabilize the lipid bilayer and lead to pore formation (Adams et al., 2014). Thus, disrupting the interaction of divalent cations with LPS could disrupt the conformational and dynamic properties of LPS, leading to membrane destabilization. However, further investigation is needed to understand the potential interactions and mechanisms of our antibodies.

One limitation of our study is that WVDC-0357 and WVDC-0496 are murine antibodies, which may have implications in efficacy and safety when translated to human use. Potential issues of murine antibodies include shorter half-lives and the possibility of triggering human anti-mouse antibody (HAMA) responses (Samaranayake et al., 2009). To address these concerns, the variable regions of these antibodies would need to be humanized and expressed recombinantly, which would minimize the risk of immunogenicity and enhance the compatibility of these antibodies with the human immune system. In addition to humanization, it is important to adhere to regulatory guidelines to assess the safety profile of these antibodies before advancing to clinical trials. According to the FDA's guidance on antibody production, pre-clinical testing would be required to evaluate potential cross-reactivity to different types of human tissue and to assess toxicity in animal models (Miele and Stein, 1997).

In summary, our study describes the development and characterization of novel mAbs directed against *P. aeruginosa* from serogroup O5. Our findings demonstrate that WVDC-0357 and WVDC-0496 are highly protective in both sepsis and pneumonia models of *P. aeruginosa* infection by promoting bacterial clearance and preventing endotoxic shock. Future work will focus on the translation of these mAbs towards clinical applications through humanization and additional translational studies. It is important to note that WVDC-0357 and WVDC-0496 only bind a subset of *P. aeruginosa* strains, and collaboration will be necessary to combine existing anti-LPS mAbs into a single therapy targeting the most prevalent serogroups of *P. aeruginosa*. Overall, these results have promising implications for the development of effective treatments against *P. aeruginosa* infections.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by West Virginia University Institutional Animal Care and Use Committee (WVU-ACUC).

Author contributions

JK, FD, and MB conceptualized and designed all experiments. WVDC-0357 and WVDC-0496 were generated and purified by JK, MM-B, and JC. Flow cytometry binding assays were conducted by JK, ES-K, and MN. Binding and functional assays were performed by JK and SM, while pharmacokinetic assays were carried out by JK and SD. Mice immunizations, administration of challenge doses, and health checks were performed by JK, MM-B, and SM. Mice necropsy and tissue processing were conducted by JK, MM-B, SM, AH, GP, SD, and EY. Histopathological analysis was completed by HM and MA. JK, FD, and MB analyzed data and composed the manuscript. All authors reviewed and edited the manuscript.

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Conflict of interest

Authors JK, MB, and FD are inventors on a pending patent application related to the sequences of WVDC-0357 and WVDC-0496.

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2023.1191806/full#supplementary-material>

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Experimental and *in silico* evaluation of *Carthamus tinctorius* L. oil emulgel: a promising treatment for bacterial skin infections

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Purpose: The current study aimed to develop a topical herbal emulgel containing *Carthamus tinctorius* L. (CT) oil extract, which has been scientifically proven for its antibacterial and antioxidant activities for the ailment of bacterial skin infections.

Method: The CT emulgel was formulated by response surface methodology (RSM) and was evaluated by various parameters like extrudability, spreadability, pH, viscosity, and antibacterial and antioxidant activities. Molecular docking was also performed using AutoDock.

Results: Among all formulated CT emulgels, F9 and F8 were optimized. Optimized formulations had shown good spreadability and extrudability characteristics. Sample F8 had % inhibition of 42.131 ± 0.335 , 56.720 ± 0.222 , and 72.440 ± 0.335 at different concentrations. Sample F9 had % inhibition of 26.312 ± 0.280 , 32.461 ± 0.328 , and 42.762 ± 0.398 at concentrations of 250 µg/ml, 500 µg/ml, and 1,000 µg/ml, respectively, which shows that both samples F8 and F9 have significant antioxidant potential. Optimized CT emulgels F8 and F9 had significant antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* at p-value = 0.00, the Emulgel-F8 shows zone of inhibition of 24 mm for *E-coli* and 19 mm for *S-aureus*. Emulgel-F9 shows zone of inhibition of 22 mm for *E-coli* and 15 mm for *S-aureus* while pure CT- Oil extract shows zone of inhibition of 25 mm for *E-coli* and 20 mm for *S-aureus* and ciprofloxacin used as standard shows 36mm zone of inhibition against both *E-coli* and *S-aureus*. The comparative investigation through molecular docking binding affinities and interactions of ligands with various target proteins provides

insights into the molecular processes behind ligand binding and may have significance for drug discovery and design for the current study.

Conclusion: The current study suggests that *C. tinctorius* L.-based emulgel has good antioxidant and antibacterial activities against *E. coli* for the treatment of bacterial skin infections.

KEYWORDS

Carthamus tinctorius L oil, extraction, anti-bacterial, anti-oxidant activity, molecular docking

1 Introduction

Transdermal drug delivery system (TDDS) defines the dosage form that distributes an optimum drug quantity across the skin. Its success overlaps the traditional processes including injectable and oral drug delivery (Kumar et al., 2016). It increases patient compliance, as the drug bypasses first-pass metabolism through TDDS. It also decreases the untoward effects of a drug raised due to an overdose of medicament (Afzal et al., 2022).

The dosage form that is comprised of combined gel and emulsion is referred to as an emulgel, which is formed by adding a gelling agent to either oil and water or water-in-oil emulsions. Emulgel shows diversity, as it has dual control on drug release due to the presence of both aqueous and non-aqueous phases in it (Yadav et al., 2016). Emulgels are widely employed for drug delivery across the skin. Their dermatological advantages are as follows: ease in the integration of drugs of hydrophobic nature, thixotropic, ungreased nature, ease in spreadability, ease in removal, emollient, stain-free, hydrophilicity, and greater shelf life with high and aesthetic appearance (Bhanu et al., 2011; Singla et al., 2012). In literature, a high rate of morbidity is reported due to skin and soft tissue infections (SSTIs) due to bacterial and fungal infections.

Microbes such as bacteria and yeasts often co-contribute these infections. An attractive approach for the treatment of superficial skin infections is the application of topical antimicrobials (Bhanu et al., 2011). The topical preparations show delivery of a high concentration of the drug across the skin on the targeted site with very few or no systemic side effects. However, few antibiotics, such as mupirocin and fluidic acid, show restrictions in their use due to their reported resistance (Sabri et al., 2016). Most *Escherichia coli* strains have been involved in various soft tissue skin infections that occur in patients of each age. Some infections recover without any treatment, but some require antibacterial treatments (Pagano et al., 2021). Almost 80% of the population uses medicinal plants in primary health care. When compared to synthetic drugs, herbal medicines have easy access, are inexpensive, and are safer (Afzal et al., 2022). Various natural or synthetic antimicrobials are being used to treat and delay the growth of different microorganisms. Antibiotics used in excess amount causes antibiotic resistance. Antibiotic resistance and toxicity may be developed by the use of

synthetic drugs. To reduce any side effects, essential oils or natural products can be used. The parameters to detect the efficacy of effective components in the natural product include its chemical properties and concentration at active component. Flavonoids, saponins, and phenolics have antimicrobial properties. The efficacy of herbal products has gained the attention of researchers, as medicines based on plants have no side effects (Kaur et al., 2021).

Carthamus tinctorius L. (CT), also called safflower or false saffron, is a medicinal plant that belongs to the family Compositae or Asteraceae. This shaft-like species typically grows in dusty Southern Asia, China, India, and Iran climates. It is cultivated mainly for its seeds, which are used as edible oil and contain many useful compounds such as flavonoids, phenolic acids, fatty acids, and alkynes, which contribute to its effect as an analgesic, anti-inflammatory, antioxidant, and antimicrobial (Abdipour et al., 2019). *In silico* studies also aid in understanding the binding energies and active sites for these biological activities (Yesilyurt et al., 2020).

Therefore, the recent research aimed to develop a new topically applied emulgel that contains oil extracted from seeds of *C. tinctorius* L. plant having both antioxidant and antibacterial properties and their *in vitro* evaluation of their antibacterial activity against *E. coli* and *Staphylococcus aureus*. The antibacterial effect was compared between the formulated products available in the market and prepared emulgel. The antibacterial and antioxidant activities were checked, and the collected data were analyzed statistically.

2 Developed methods and materials

2.1 Chemicals

C. tinctorius oil, Carbopol 940 polymer, polyethylene glycol, methylparaben, Tween 20, Span20, *n*-hexane, methanol, propylene glycol, triethanolamine, NaOH, and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany), as well as distilled water, Mueller-Hinton agar (OXOID CM0337), and Nutrient broth (OXOID CM0001). All other ingredients were of analytical grade.

2.2 Collection, extraction, and isolation of oil from the plant

CT plants were collected from Pakistan's tropical regions. The oil from the CT plants' seeds was extracted using a Soxhlet apparatus (Pyrex, Germany). For extraction, *n*-hexane, a hydrocarbon having a boiling point of 68.7°C, was chosen as the preferred solvent. After extraction, oil was obtained by removing the solvent using a rotary evaporator (Javed et al., 2018a). After that, the solubility of CT oil was determined in methanol, *n*-hexane, and buffer solution (pH 5.5).

2.3 Design expert for experiment

The experimental permeation enhancer concentration like propylene glycol (PG) and polyethylene glycol (PEG) on emulgel was assessed using Design Expert (version 7.0.3). The quantities of PG and PEG were tested at three altitudes using a central composite rotatable design (CCRD), as indicated in Table 1, with other variables remaining constant throughout the study while taking into account the principal point of 0, 0 in quintuplicate.

2.4 Preparation of emulgel

As shown in Table 2, 100 g of the *C. tinctorius* L. oil-based emulgel was made using various concentrations of PG and PEG.

TABLE 1 Factor arrangements: (a) concrete elements analysis with coded levels and (b) experimental design range.

Serial no. (a)	Coded level factors		PG	PEG
	X ₁	X ₂		
F1	0	0	10	3
F2	0	+1	10	4
F3	+1	0	13	3
F4	0	−1	10	2
F5	−1	0	6	3
F6	+1	+1	13	4
F7	−1	−1	6	2
F8	+1	−1	13	2
F9	−1	+1	6	4
F10	0	0	6	4
F11	0	0	6	4
F12	0	0	6	4
F13	0	0	6	4
(b).				
Coded level	−1		0	+1
X ₁ (g) PG	6		10	13
X ₂ (g) PEG	2		3	4

PG, propylene glycol; PEG, polyethylene glycol.

Initially, the gel phase was made with the necessary Carbopol 940 concentration to water at an appropriate speed with continuous stirring by a mechanical stirrer. Span20 was mixed with liquid paraffin, following the addition of CT oil to form the oil phase. Tween 20 was dissolved in distilled water to form an aqueous phase, and then polyethylene glycol-1000 and methylparaben were added. Then, both phases were heated to 70°C to 80°C, cooled at room temperature, and mixed to make emulsion. To develop emulgel, the emulsion was diluted with gel in a ratio of 1:1 while swirling gently (Javed et al., 2018b). Figure 1 shows the physical appearance of the formulated emulgel.

2.5 Physicochemical evaluation

The visual appearance, color, homogeneity, consistency, and pH of each CT-emulgel formulation were evaluated (Mottaleb et al., 2007). At 25°C, Brookfield viscometer with spindle assessed CT-emulgel formulation viscosity (Javed et al., 2018b).

2.5.1 Skin irritation test

Applying CT-emulgel formulations on well-shaven rat skin, the skin irritation test was monitored for 24 h to observe unfavorable changes in morphology and color.

2.5.2 Spreadability

CT-emulgel mixture with a mass of 0.1 g was placed on the transparent 1.6-cm-diameter center of a glass slide, which was then squeezed for 6 min by placing a second glass slide on it. Each CT emulgel had a centimeter-long circled spreading portion (*n* = 3). The following formula was used to calculate the average (Javed et al., 2018b).

$$S = [M \times L/T], \quad (1)$$

where *M* stands for the weight (g) bent to the top slide, *L* for the length (cm), and *T* for the time (seconds).

2.5.3 Extrudability

The extrudability test of each CT emulgel determines if it flows freely from the collapsible tubes. The extrudability of CT emulgel was determined using a hardness tester. CT emulgel with a mass of 5 g was placed inside a collapsible tube. A pressure of 1 kg/cm² was applied for 30 seconds by holding the tube with a plunger. Emulgel extruded from each tube was measured (Javed et al., 2018b).

2.6 Fourier transform infrared spectroscopy

By conducting Fourier transform infrared (FTIR) spectroscopy of polymers, drugs, and drug-loaded emulgels, structural factors such as polymer–drug interactions of CT-emulgel formulations were confirmed (Chakole et al., 2009). FTIR spectroscopy analyzed the values of several functional groups.

TABLE 2 *Carthamus tinctorius* L. oil-based emulgel 100-g preparation.

Formulations	C. <i>tinctorius</i> oil % w/w	Carbopol 940 (% w/w)	X ₁ = propylene glycol (%w/w)	X ₂ = PEG (% w/w)	Tween 20 (% w/w)	Span20 (%w/w)	Methyl paraben (%w/w)	Triethanolamine	Distilled Water
F1	2.5	2	10	3	0.30	0.99	0.03	0.02	81.5
F2	2.5	2	10	4	0.30	0.99	0.03	0.02	80.15
F3	2.5	2	13	3	0.30	0.99	0.03	0.02	81.5
F4	2.5	2	10	2	0.30	0.99	0.03	0.02	82.7
F5	2.5	2	6	3	0.30	0.99	0.03	0.02	85.15
F6	2.5	2	13	4	0.30	0.99	0.03	0.02	85.15
F7	2.5	2	6	2	0.30	0.99	0.03	0.02	77.15
F8	2.5	2	13	2	0.30	0.99	0.03	0.02	79.15
F9	2.5	2	6	4	0.30	0.99	0.03	0.02	84.15
F10	2.5	2	6	4	0.30	0.99	0.03	0.02	84.15
F11	2.5	2	6	4	0.30	0.99	0.03	0.02	84.15
F12	2.5	2	6	4	0.30	0.99	0.03	0.02	84.15
F13	2.5	2	6	4	0.30	0.99	0.03	0.02	84.15

2.7 Stability studies and statistical analysis

The stability compartment was maintained at 40.1°C to check the stability of all CT emulgels for 6 months. After 0 months to 6 months, different physical aspects of CT emulgels, such as viscosity, pH, and drug contents, were examined (Mathias and Hussain, 2010). With Microsoft Excel 2013 statistical data, the mean and standard deviation were analyzed. Thirteen distinct formulations were compared statistically using analysis of variance (ANOVA) (Khan et al., 2020).

2.8 Drug release of CT emulgels (*in vitro*)

The *in vitro* drug release study of CT emulgels utilized the dissolution apparatus [USP type II]. The CT oil extract was administered as a pharmaceutical agent in phosphate-buffered saline (PBS), with a release medium at a pH of 5.5 and a temperature of 37.5°C. This was achieved by encapsulating 1 g of CT emulgel within a cellophane membrane and attaching it to the paddle of a dissolution apparatus using a thread. A volume of 500 ml of PBS was employed, while the paddle rotation speed

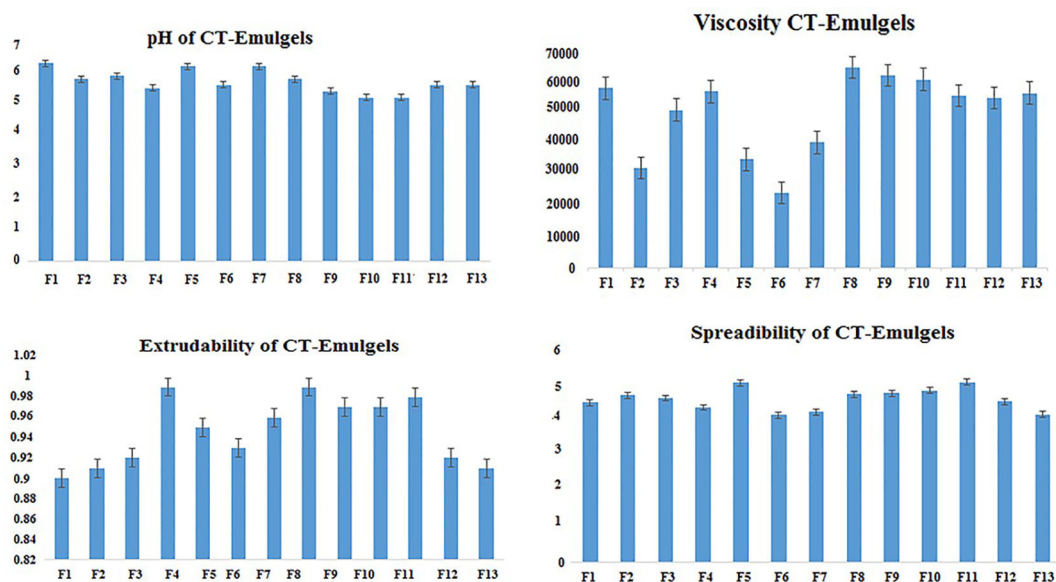


FIGURE 1
Physicochemical characteristics of CT emulgels. CT, *Carthamus tinctorius* L.

ranged from 85 to 100 revolutions per minute (RPM). From the release media, 5 ml of the sample was removed after a predetermined amount of time and replaced with the same volume of fresh media (Shah et al., 2009). The collected sample was examined using a UV spectrophotometer (PerkinElmer, Waltham, MA, USA) to ascertain its CT oil concentration at 256 nm (Akaike, 1998). A calibration curve was applied to determine the amount of CT oil extract present in each batch of CT emulgel (Nisar Hussain Shah, 2012).

2.9 Thermal analysis

Thermal behavior of optimized emulgels F8 and F9 was recorded by thermogravimetric analysis (TGA) to analyze the physical state of CT oil present in emulgel formulations. The sample sizes used were 14.7 mg and 16.6 mg for F8 and F9, respectively. Under a nitrogen environment, heat stress was applied at a rate of 10°C/min from 20°C to 350°C, and thermogram peaks were recorded (Devi et al., 2021).

2.10 Scanning electron microscopy analysis

Scanning electron microscopy (SEM) was used to analyze the surface morphology and superficial shape of emulgels. The sampling for SEM was performed by attaching emulgels on metal slabs using adhesive tape and kept in a vacuum chamber for drying. A layer of 10-nm thickness consisting of a dense gold coating was subsequently applied using a sputter coater. Subsequently, a high-resolution SEM analysis was conducted to detect and examine the sample (Ambala and Vemula, 2015).

2.11 Antioxidant activity

The radical scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was calculated to check the antioxidant activity of CT emulgels F8 and F9. At various concentrations (250 µg/ml, 500 µg/ml, 1,000 µg/ml), 1 ml from 0.1 mM of DPPH solution was prepared in ethanol and then mixed with 3 ml of the tested compounds in ethanol formulated by successive dilution. The prepared mixture was placed at room temperature of approximately 22°C and shaken for 30 min, and then the reaction mixture's absorbance was investigated using a UV spectrophotometer at 517 nm. Different concentrations were used to detect the inhibition concentration for 50% of DPPH free radical (IC₅₀). Ascorbic acid was used as a standard antioxidant agent (Ambala and Vemula, 2015; Méndez et al., 2018). The following equation was used to calculate radical scavenging activity.

% Radical Scavenging = $(1 - \text{Absorbance of sample} / \text{Absorbance of Control}) \times 100$.

The absorbance of control is the absorbance of pure DPPH solution.

2.12 Antibacterial activity

2.12.1 Media preparation

Mueller–Hinton is a commonly used agar medium in microbiology for antimicrobial susceptibility testing. This agar media method was employed for the preparation of the media. To prepare the media, 38 g of agar was added to 1 L of water and then heated to dissolve it completely after autoclaving for 15 min at 121°C. This media was utilized for the agar well diffusion procedure. A nutrient broth media was created to prepare the bacterial solution by adding 13 g of powder to 1,000 ml of distilled water. After thorough mixing, this was sterilized by autoclaving for 15 min at 121°C (Ermawati et al., 2020).

2.12.2 Experimental microbes

Different microbial strains, *S. aureus* (ATCC-2529) and *E. coli* (ATCC-27853) were used.

2.12.3 Preparation of bacterial solution

The freshly prepared culture of *S. aureus* and *E. coli* was selected. In the next step, nutrient broth media was inoculated with bacterial colonies. To obtain precise results, nutrient media was inoculated with maximum colonies, and for each *S. aureus* and *E. coli*, an optical density greater than 1 was found.

2.12.4 Plating

Petri dishes were used for plating and solidified by pouring liquefied Mueller–Hinton agar (11–14 ml). Then, the plates were labeled (Alexander et al., 2013).

2.12.5 Serial dilution

To obtain a precise number of bacteria per 1 ml, serial dilutions were prepared as necessary to attain 1,000 bacteria per 100 µl. For serial dilution, there were five Falcon test tubes for each strain, and a total of 10 test tubes were used. A nutrient broth of 9 ml was added to each test tube and then labeled as 101, 102, 103, 104, and 105 for each strain of bacteria. Ten Falcon tubes were used for each strain of bacterial dilution. Nutrient broth with volume of 9 ml was added to each Falcon tube and then labeled as 101, 102, 103, 104, and 105 for each bacterial strain. From bacterial solution, 1 ml was used for Falcon tube 5, labeled as 105, and mixed and added to Falcon tube 104. The same procedure was repeated until the last one was finished, which was Falcon tube 4 (as Falcon tube 5 contained 105 per ml, same as the others). Dilution 101 with volume of 100 µl was taken via micropipette and poured on plates containing Mueller–Hinton agar, as this contained 1,000 bacteria (RaMalingam et al., 2022).

2.12.6 Spreading and well formation

In the next step, 100 µl of serial dilution was selected and poured into a petri dish labeled as 4, designated for specific bacteria. Following that, with the help of swabs and loops, lawn and wells were made in petri dishes, respectively. A cotton swab was used to

spread bacterial solution in multiple directions to prepare equally dispensed bacterial loon. According to the arrangement of sample per plate, 1-ml autoclaved micropipette tips were used to spread wells in the agar media.

2.12.7 Sample loading and incubation

In this step, a micropipette in the range of 10 μ l to 200 μ l was used to pour 100 μ g into the well. The sample was incubated for 24 h at 37°C. CT emulgel with a mass of 100 μ g containing 2 μ g of the extract was loaded in wells. The standard used was ciprofloxacin 10 μ g (Wulansari et al., 2017; Chauhan, 2021). The plates were removed from the incubator after 24 h, and the diameter of the inhibition zone was measured.

2.13 Minimum inhibitory concentration and minimum bactericidal concentration determination

After 48-h incubation, the minimum inhibitory concentration (MIC) determined was the minimum concentration that shows no turbidity, and the minimum bactericidal concentration (MBC) determined was the minimum concentration that produces not even a single colony of bacteria. With the use of MBC and MIC, serial dilutions of oil were primed. To make serial dilutions, MIC and MBC values were prepared using the microliter broth method; with this method, a methanol extract of 10 μ g/100 μ l was prepared, and MIC and MBC for bacterium *E. coli* ranges were determined using the microliter broth dilution method. This method filled the wells with 100 μ l of nutrient broth to prepare serial dilutions. The ultimate oil extract concentrations in the wells were 10 μ g/ml, 5 μ g/ml, 2.5 μ g/ml, 1.5 μ g/ml, 1.25 μ g/ml, 0.625 μ g/ml, 0.312 μ g/ml, 0.156 μ g/ml, 0.078 μ g/ml, and 0.039 μ g/ml. After this, 100 μ l of *E. coli*-based bacterial sample was added to well numbers 1–10, taken from 108 Falcon tube dilution. Then, the last two wells were marked as negative and positive controls. The negative control had only 100 μ l of methanol extract solution, while the positive control had 100 μ l of bacterial solution. These controls were then incubated for 24 h at 37°C. From this, MIC and MBC values were identified. In order to confirm bacterial absence, 100 μ l was taken from the wells marked to have MIC and MBC values, poured into two separate nutrient agar plates, and incubated at 37°C for 18–42 h (Afzal et al., 2022).

2.14 Molecular docking methodology

The RCSB protein data repository included urate oxidase and *Helicobacter pylori* urease crystalline structures for virtual screening. Molecules were docked using MGLTools, AutoDock4, and Autogrid4 binary files. International Union of Pure and Applied Chemistry (IUPAC) designations were used to draw the ligand structures in ChemDraw Ultra.

The RCSB protein data repository included urate oxidase and *H. pylori* urease crystalline structures for virtual screening (Alawlaqi et al., 2023; Jabeen et al., 2023) (1R4U and 4HI0). Molecules were

docked using MGLTools, AutoDock4, and Autogrid4 binary files (Morris et al., 2009). The 2D structures were depicted using the ChemDraw Ultra software (Draw, 2010), utilizing their IUPAC names. Subsequently, the Chem 3D Pro software (CambridgeSoft, 2009) was employed to perform energy minimization. The ligand structures underwent optimization and were subsequently saved in the sdf format. The Open Babel graphical user interface (GUI) software was utilized to convert these structures into the pdbqt format compatible with AutoDock. The pre-processing and visualization of proteins were conducted using the Discovery Studio Visualizer software developed by BIOVIA (Visualizer, 2005). The protein molecules in BIOVIA's Discovery Studio Visualizer were devoid of heteroatoms, co-crystal ligands, and solvent molecules following a deletion process. After adding polar hydrogen and Kollman charges to each atom, the protein structures were saved in pdbqt format for autodocking (Ferreira et al., 2015). Structure-based virtual screening was performed using AutoDock4. The grid parameter file was created using the co-crystal ligand's grid box dimensions (Zentgraf et al., 2007). The docking parameter files were generated using LGA and AutoDock4Zn (Morris et al., 2009). In order to ensure the reliability of the study, a total of 50 poses and 300 individuals were included in the analysis. The active sites of both proteins were subjected to docking analysis with the ligand library (Yusuf et al., 2008).

3 Experimental results

3.1 Solubility studies

C. tinctorius L. oil's solubility was tested using PBS at pH 5.5 and was found to be 0.004–0.003 mg/ml. Its solubility in hexane was 0.098–0.01 mg/ml, while it was 0.092–0.01 mg/ml in methanol. These findings demonstrated the distinct solubility of *C. tinctorius* L. oil in hexane to other solvents.

3.2 Physicochemical characteristics

Table 3 contains the physical characteristic analysis of the prepared *C. tinctorius* L. oil-based emulgels, including their spreadability, homogeneity, consistency, thickness, and pH.

3.2.1 Stability testing and Draize's skin irritation test

According to the outcomes of stability testing, all CT-emulgel formulations were carried out by keeping them all for 6 months at a temperature of 40°C $\pm$ 1°C in a stability chamber. All CT-emulgel formulations had satisfactory appearances, and no significant alterations in drug content, drug release behavior, viscosity, pH, or moisture content were found. All CT-emulgel formulations underwent a 30-day skin irritation test using human volunteers. According to the findings, there were no skin lesions, irritations, or scratches. Similar findings were reported in a previously reported article (Javed et al., 2018a; Javed et al., 2018b).

TABLE 3 Physical characteristics of formulated CT emulgels (n = 3, mean ± SEM).

Formulations	pH	Spreadability (mean ± SEM)	Extrudability (mean ± SEM)	Viscosity (centipoise) (mean ± SEM)
F1	6.2 ± 0.033	4.420 ± 0.006	0.910 ± 0.006	53,500.000 ± 0.006
F2	5.7 ± 0.033	4.617 ± 0.003	0.923 ± 0.009	29,466.667 ± 0.003
F3	5.8 ± 0.033	4.527 ± 0.009	0.920 ± 0.006	47,000.000 ± 0.009
F4	5.6 ± 0.033	4.270 ± 0.006	0.960 ± 0.021	52,300.000 ± 0.006
F5	6.1 ± 0.033	4.970 ± 0.006	0.937 ± 0.006	31,898.000 ± 0.006
F6	5.5 ± 0.033	4.073 ± 0.003	0.927 ± 0.003	23,110.000 ± 485.215
F7	6.3 ± 0.033	4.160 ± 0.006	0.947 ± 0.009	37,236.333 ± 68.216
F8	5.7 ± 0.033	4.660 ± 0.006	0.973 ± 0.012	59,400.000 ± 152.753
F9	5.3 ± 0.033	4.677 ± 0.003	0.947 ± 0.012	57,200.000 ± 57.735
F10	5.3 ± 0.033	4.747 ± 0.003	0.950 ± 0.010	54,100.000 ± 1,154.701
F11	5.1 ± 0.033	4.977 ± 0.003	0.970 ± 0.006	51,500.000 ± 173.205
F12	5.5 ± 0.033	4.443 ± 0.007	0.947 ± 0.015	50,456.667 ± 110.504
F13	5.5 ± 0.033	4.080 ± 0.006	0.940 ± 0.015	52,233.333 ± 88.192

CT, *Carthamus tinctorius* L.

3.2.2 Spreadability and extrudability

According to the data presented in Table 3 and Figure 1, the spreadability range of all CT emulgels prepared with Carbopol 940 as the gelling agent was observed to be approximately 4.3 ± 0.1 g-cm/s to 4.7 ± 0.1 g-cm/s. CT emulgel F9 spread easily in comparison to all other preparations (Mohite and Salunkhe, 2019). The extrudability results showed that less viscous CT emulgel had acceptable tube flow ability, while more viscous CT-emulgel preparations were difficult to extrude from tubes. The extrudability ranges were 1.272 ± 0.01 , 0.961 ± 0.01 , and 0.866 ± 0.01 . CT emulgel F9 demonstrated extrudability of 0.961 ± 0.01 g/cm. The results are given in Table 3.

3.3 RSM optimization result in mathematical modeling

Multiple linear regression analysis (MLRA) was employed to produce a mathematical relationship based on a polynomial equation. The positive and negative significant effects have demonstrated the comparative impacts of each factor on a given response of CT emulgels. Negative numbers represent antagonistic effects on each response, while positive values demonstrate synergistic effects.

p-Value (p 0.05) for answer response Y1 indicates linear contributions. A (PG) and B (PEG) both exhibited negligible antagonistic and cross-product effects. According to ANOVA (Table 4), AB (PG with PEG) demonstrated a significant antagonistic reaction, A2 (a quadratic contribution) demonstrated a significant synergistic response, and B2 demonstrated a synergistic non-significant effect. Table 5 demonstrates multiple linear regression analysis. Here, a polynomial equation has coded factors.

$$Y_1 = b_0 + b_1A + b_2B + b_{12}AB + b_1^2A^2 + b_2^2B^2, \quad (2)$$

$$Y_1 = 75.59 - 0.38A - 1.65B - 4.02AB + 4.74A^2 + 1.08B^2, \quad (3)$$

According to the equations, it was determined that while “B” had a significant negative antagonistic impact on the release of CT oil in PBS, “A” had a relatively minor negative antagonistic effect at pH 5.5. Both of the variables (AB) demonstrated potent combined effects that demonstrated adverse (antagonistic) consequences. Propylene glycols (A2), which had a high positive (synergistic) effect, had a quadratic relationship with PEG, which is a weak positive (synergistic) relationship. Propylene glycol (A) $[-0.38A + 4.74A^2]$ showed that the percentage of drug release increased when PG concentration was higher, while the concentration of PG fell when PG concentration was lower. According to previously published research (Javed et al., 2018a), the percentage of drug release rose with higher PEG concentrations and decreased with lower PEG concentrations. This is shown by the equation PEG (B) $[-1.65B + 1.08B^2]$. Figure 2 displays the 3D surface plots and the contour plot. The CT-emulgel F9 formulation was chosen for further research on either humans or animals since the findings of the response surface methodology (RSM) data analysis revealed that it had the largest amount of drug release when compared to other formulations.

3.4 Stability testing and Draize’s skin irritation test

According to the stability findings, all CT-emulgel formulation preparations were carried out by keeping them all for 6 months at a temperature of $40^\circ\text{C} \pm 1^\circ\text{C}$ in a stability chamber. The findings demonstrated that all CT emulgels have satisfactory outward appearances, and no significant alterations in drug content, drug

TABLE 4 Analysis of multiple linear regression analysis (MLRA).

Regression coefficient	Y ₁
Model	Quadratic
Intercept	75.59
A	−0.3817
B	−1.65
AB	−4.02
A ²	4.74
B ²	1.08
Model (p-value)	0.01
Coefficient value %	1.79
R ²	0.92
Adjusted R ²	0.85
Predicted R ²	0.74
Adjusted precision	11.60
Standard deviation	1.43
Mean	79.55
F-value	12.64

release behavior, viscosity, pH, or moisture content were found. All CT-emulgel preparations underwent a 30-day skin irritation test using human volunteers. According to the findings, there were no skin lesions, irritations, or scratches. Similar findings were reported in previously reported articles (Hadgraft and Lane, 2016).

3.5 In vitro drug release study

In vitro drug release profiles of CT emulgels are shown in Figure 3. Drug release was observed in optimized CT-emulgel formulations in the order of F9 > F8. In comparison to other emulgel preparations, the F9 was thought to have the highest CT release (90%). Various factors, such as gelling agents, emulsifying agents, and formulation viscosity, may affect drug release. In our

study, polyethylene glycol was used as a penetration enhancer, and by increasing polyethylene concentration, drug release was also increased (Burki et al., 2020).

3.6 Fourier transform infrared spectroscopy

The analysis of FTIR spectra of the produced CT emulgels, *C. tinctorius* L. oil, and Carbopol 940 revealed several interesting findings, as shown in Figure 4. No significant differences were observed in the FTIR spectra of the CT emulgels, *C. tinctorius* L. oil, and Carbopol 940. This suggests that the formulation process and the addition of excipients did not cause major changes in the chemical composition of these components. In Figure 4, the amide group (−NH) in the CT emulgel exhibited broad peaks in the range of 2,800–3,800/cm. These peaks were wider than those observed in pure drug polymer. The broadening of the peaks indicates potential interactions between the amide group and other components in the emulgel formulation. The C=C alkene group showed distinct peaks in the range of 1,440–1,650/cm. According to a recent article (Javed et al., 2018a), this peak was more pronounced in the CT-emulgel formulation compared to the polymer and CT oil alone. The presence of a distinct peak in this range consistently across all formulations suggests the presence of the alkene group in the emulgel and strong bond interactions within this group. Peaks in the range of 1,120–1,180/cm were observed due to the presence of phenyl groups, while peaks in the range of 700–733/cm were attributed to the presence of hydroxyl (O–H) groups. These findings indicate the presence of specific functional groups in the CT-emulgel formulation. The stability of CT oil in all CT-emulgel formulations, including Carbopol 940, polymers, and other excipients, was determined to be satisfactory (Ayoub et al., 2015; Abdipour et al., 2019). This implies that the formulation is effective in preserving the stability of the CT oil even in the presence of other ingredients. The results are significant, as they provide valuable insights into the chemical interactions and stability of the CT-emulgel formulation. The broadening and shifting of peaks in the FTIR spectra indicate the occurrence of chemical interactions between various components in the emulgel. These findings

TABLE 5 Variance analysis of *Carthamus tinctorius* oil-based emulgel.

Source	Sum of square	Df	Mean square	F-value	p-Value	
Model	134.57	5	26.91	13.25	0.006	Significant
A-PG	0.98	1	0.98	0.48	0.518	Non-significant
B-PEG	18.25	1	18.25	8.98	0.0302	Non-significant
AB	65.23	1	65.23	32.12	0.0024	Significant
A ²	50.92	1	5.92	25.07	0.0041	Significant
B ²	2.81	1	2.81	1.38	0.2926	Non-significant
Residual	10.15	5	2.03	–	–	
Lack of fit	2.15	3	0.72	0.18	0.9023	Non-significant

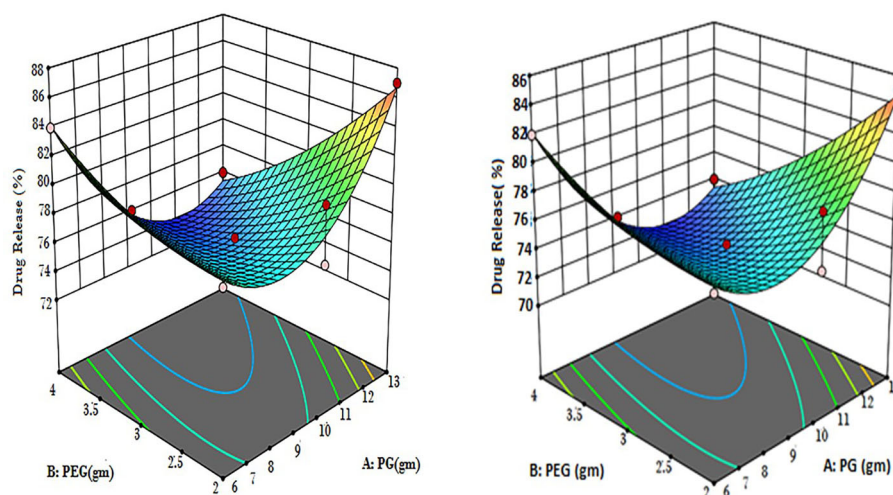


FIGURE 2
Contour 3D surface plots of optimized CT emulgels F8 and F9. CT, *Carthamus tinctorius* L.

support the suitability of the chosen formulation for the intended purpose.

3.7 Thermal analysis

The TGA measurement was used to check the thermal stability of emulgel within the 25°C–350°C temperature range. The TGA plot of the emulgel is presented in Figure 5. The oil's flash point is 110°C, indicating minimal degradation of the emulgel at this temperature and suggesting a stable formulation.

3.8 SEM analysis

The SEM analysis of the emulgel reveals that the surface exhibits an irregularly shaped structure, characterized by the presence of

cracks and wrinkles. This observation is depicted in Figure 6, which illustrates samples F8 and F9. Additionally, the image reveals the presence of minute porous channels, which can be attributed to the removal of water molecules during the preparation process. The presence of various irregularly sized pockets of CT oil on the surface may be attributed to the phenomenon of polymer cross-linking within emulgel formulations.

3.9 Measurement of DPPH assay for antioxidant activity

The antioxidant activity of optimized CT emulgels was also checked using DPPH free radical scavenging assay. In this assay, the protons were accepted as free radicals from the antioxidant substrate, decreasing its absorbance, which was reserved as an amount of radical scavenging.

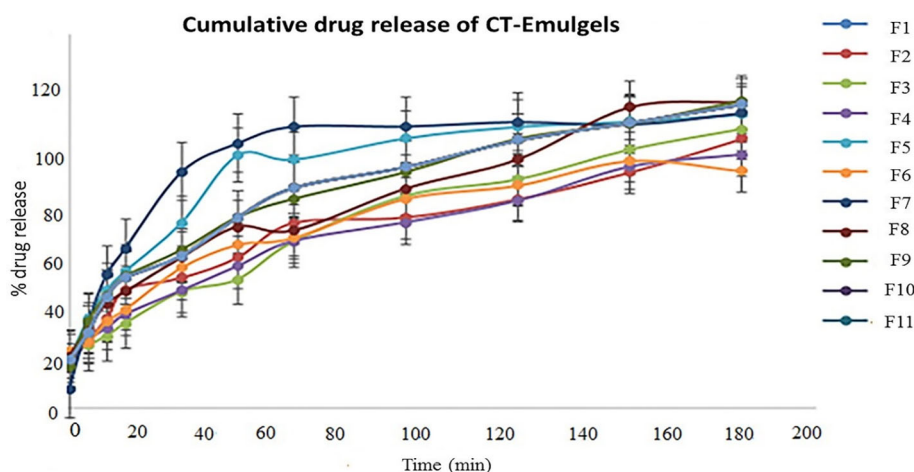


FIGURE 3
Cumulative % drug release of CT-emulgel formulations. CT, *Carthamus tinctorius* L.

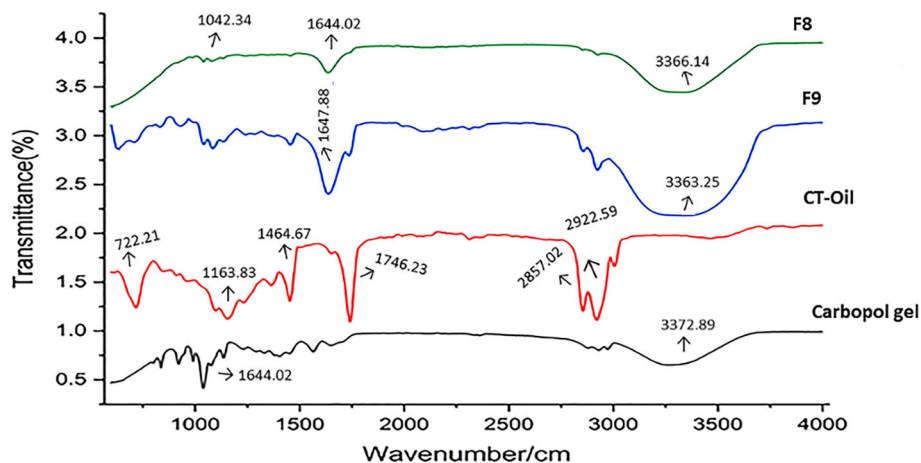


FIGURE 4

FTIR peaks of CT emulgels F8 and F9, *Carthamus tinctorius* L. oil, and Carbopol 940. FTIR, Fourier transform infrared; CT, *Carthamus tinctorius* L.

Sample F8 had % inhibition of 42.131 ± 0.335 , 56.720 ± 0.222 , and 72.440 ± 0.335 at different concentrations. Sample F9 had % inhibition of 26.312 ± 0.280 , 32.461 ± 0.328 , and 42.762 ± 0.398 at concentrations of 250 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$, and 1,000 $\mu\text{g/ml}$, respectively. The standard drug used had % inhibition of 24.030 ± 0.578 , 26.030 ± 0.619 , and 33.607 ± 0.294 at concentrations of 250 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$, and 1,000 $\mu\text{g/ml}$, respectively, which shows that both samples F8 and F9 have significant antioxidant potential. The radical scavenging activity of optimized CT emulgels F8 and F9 is also shown in Figure 7 and Table 6.

3.10 Antibacterial activity

As a result of the study, it was found that *C. tinctorius* L. oil-based emulgels F8 and F9 possess antibacterial action against different test strains. The results obtained in terms of the

diameter of the zone of inhibition (mm) are summarized in Figure 8. Bacterial strains like *E. coli* and *S. aureus* were inhibited by emulgel formulations.

To determine the antibacterial activity of various formulations of emulgel, the diameter and zone of inhibition (mm) were measured and compared. The specified amount of emulgel was taken in the recent research. It was observed that CT emulgel had significant antibacterial activity against *E. coli* and *S. aureus* as shown in Figure 8. The compared results of CT emulgel F8 and CT emulgel F9 indicate that samples F8 and F9 had maximum activity against *E. coli* with a small zone of inhibition toward *S. aureus*. However, pure CT oil shows a significant zone of inhibition against both bacterial strains.

A p-value of 0.00, which is less than 0.05, means that there is a significant difference existing among the means as shown in Table 7. The statistical analysis was conducted using the MINITAB 17 statistical package software, specifically employing the one-way

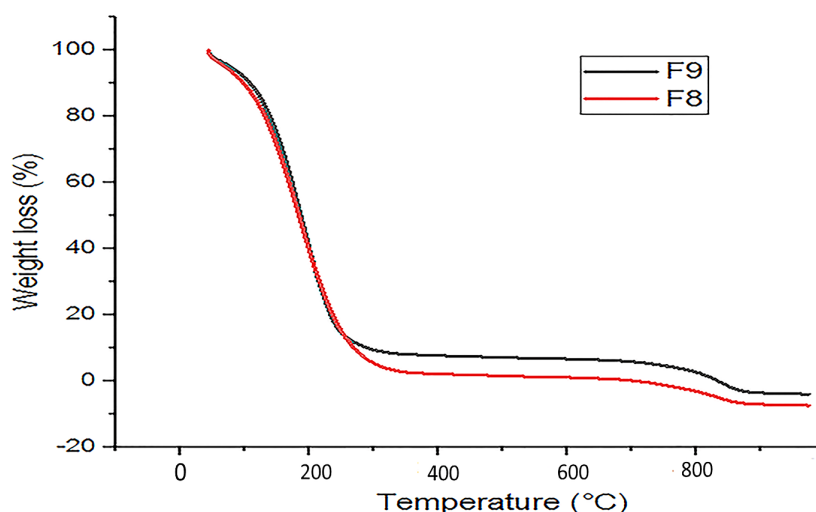


FIGURE 5

Thermal analysis of optimized CT emulgels F8 and F9. CT, *Carthamus tinctorius* L.

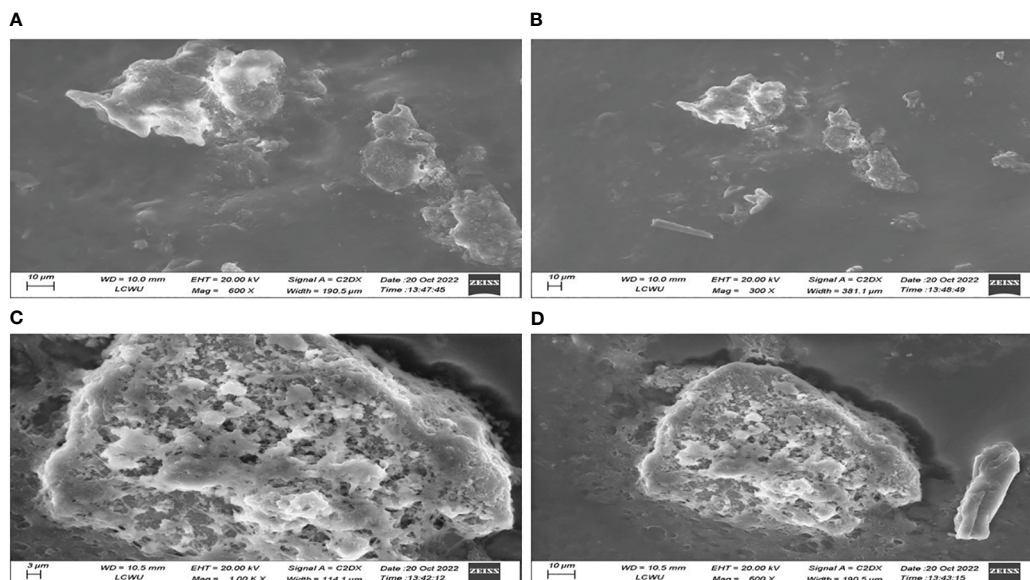


FIGURE 6

SEM investigation of CT emulgels F8 (A, B) and F9 (C, D) at different magnifications. SEM, scanning electron microscopy; CT, *Carthamus tinctorius* L.

ANOVA method ($p < 0.05$). All formulations collectively exhibited a statistically significant antibacterial effect against all tested bacteria, as indicated by a p-value of 0.00 in Table 7.

Here, F8 shows more mean area in the zone of inhibition as compared to F9, but standard drug ciprofloxacin shows a greater zone of inhibition as compared with other formulations.

Emulgel F8 shows a zone of inhibition of 24 mm for *E. coli* and 19 mm for *S. aureus*. Emulgel F9 shows a zone of inhibition of 22 mm for *E. coli* and 15 mm for *S. aureus*. Pure CT oil extract shows the zone of inhibition of 25 mm for *E. coli* and 20 mm for *S. aureus*. Ciprofloxacin used as standard shows a 36-mm zone of inhibition against both *E. coli* and *S. aureus* as shown in Tables 8, 9 and Figure 9.

MIC and MBC values were 0.03 µg/100 µl and 0.06 µg/100 µl, respectively, for *E. coli* and 0.25 µg/100 µl and 0.05 µg/100 µl, respectively, for *S. aureus* as shown in Table 10. The graphical representation of the zone of inhibition against both *S. aureus* and *E. coli* is also shown in Figure 9 and Table 10.

4 In silico results

The crystalline structures of urate oxidase, known for its antioxidant properties, and *H. pylori* urease, recognized for its antibacterial activity, were obtained for virtual screening. Protein Data Bank (PDB) identifiers 1R4U and 4HI0 were utilized to access these structures from the RCSB

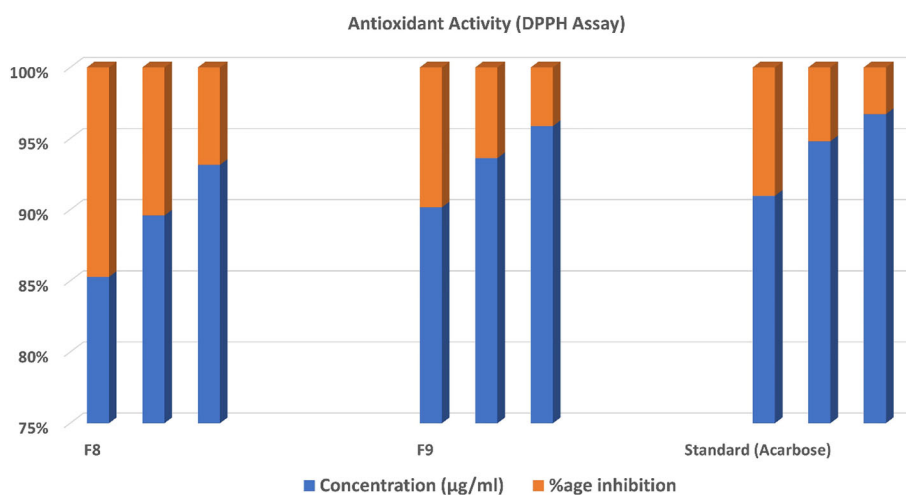


FIGURE 7

Radical scavenging activity of CT emulgels F8 and F9. CT, *Carthamus tinctorius* L.

TABLE 6 Antioxidant activity (DPPH assay).

Sample		Concentration ($\mu\text{g/ml}$)	Absorption	% inhibition $\pm$ SEM	IC ₅₀ ($\mu\text{g/ml}$)
F8	1	250	1.964	42.131 $\pm$ 0.335	375.15
	2	500	1.449	56.720 $\pm$ 0.222	
	3	1,000	0.917	72.440 $\pm$ 0.335	
F9	1	250	2.5132	26.312 $\pm$ 0.280	132.8
	2	500	2.2791	32.461 $\pm$ 0.328	
	3	1,000	1.9701	42.762 $\pm$ 0.398	
Standard (acarbose)	1	250	2.598	24.030 $\pm$ 0.578	123.583
	2	500	2.509	26.030 $\pm$ 0.619	
	3	1,000	2.282	33.607 $\pm$ 0.294	

protein data library. The crystal structures of these proteins were demonstrated in their native state, with a resolution of 2.20 Å. Additionally, the crystal structures of these proteins were examined in their binding state with the investigated ligands 1_KA–10_KA. The results presented in Tables 11, 12 demonstrate the binding affinity, hydrogen bonding, hydrophobic interactions, and electrostatic interactions between the active site pocket amino acids and the proteins and the corresponding distances measured in angstroms.

In this comparative analysis, Table 11 shows the binding affinities and interactions of 10 ligands (1_KA to 10_KA) with protein 1R4U. The binding affinity (ΔG) is a measure of how tightly a ligand binds to a protein, with more negative values indicating stronger binding. The interactions include hydrogen bonding, hydrophobic interactions, and electrostatic interactions, which play crucial roles in stabilizing ligand–protein complexes. Among the ligands, 3_KA (Figure 10) shows the highest binding affinity of -8.3 kcal/mol, indicating that it forms a

strong and stable complex with the protein. This ligand interacts through hydrogen bonding with THR173 and GLU259. The involvement of multiple hydrogen bonds likely contributes to its high binding affinity. Additionally, the hydrophobic interactions with LEU170 and PHE258 further stabilize the complex. In contrast, ligands 4, 5, 6, 8_KA, and 10_KA exhibit weaker binding affinities ranging from -4.3 kcal/mol to -5.8 kcal/mol. These ligands may have limited interactions with the protein, which could result in weaker binding. Notably, 4_KA has only one hydrophobic interaction with ARG176, which might explain its lower affinity when compared to others. Ligands 1_KA (Figure 11), 2_KA (Figure 12), 7_KA, and 9_KA fall in between, with binding affinities ranging from -4.4 kcal/mol to -7.4 kcal/mol. However, they exhibit multiple hydrogen bonding interactions, and their hydrophobic and electrostatic interactions appear to be less pronounced, contributing to their moderate binding affinities.

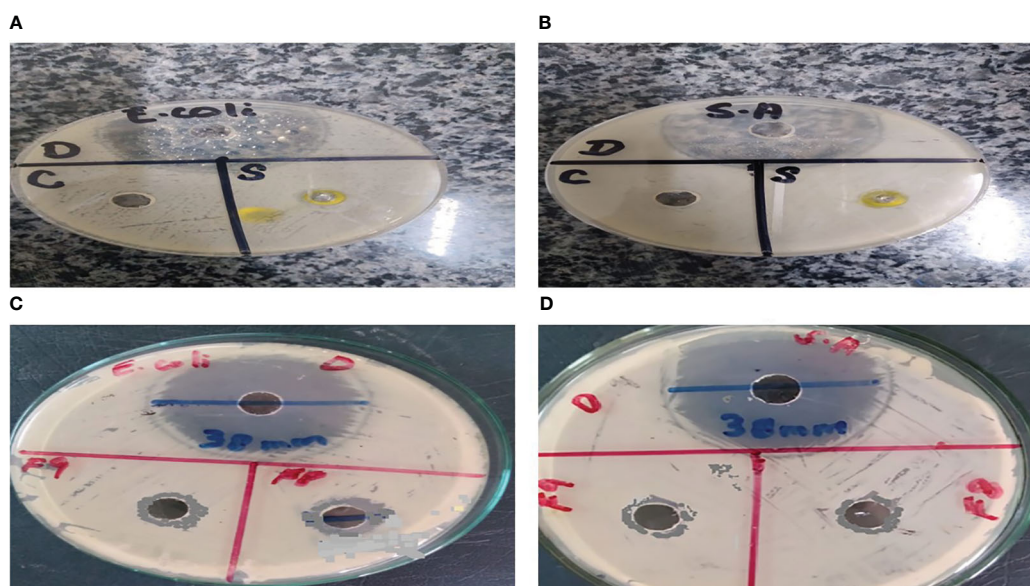


FIGURE 8

Antibacterial activity of *Carthamus tinctorius* L. (CT) oil against (A) *Escherichia coli* and (B) *Staphylococcus aureus*. (C) CT emulgels F8 and F9 against *E. coli*. (D) CT emulgels F8 and F9 against *S. aureus*.

TABLE 7 Variance scores of CT emulgels F9 and F8 against *Escherichia coli*.

Source	DE	Adj SS	Adj MS	F-value	p-Value
CI	2	366.22	183.111	274.67	0.000
Error	6	4.00	0.66	–	–
Total	8	370.22	–	–	–

CT, *Carthamus tinctorius* L.

TABLE 8 Mean extract zone of inhibition against *Escherichia coli* (n = 3).

C1	Bacterium	Mean	Standard deviation	95% CI
F8	<i>Escherichia coli</i>	24.333	0.577	(23.180, 25.487)
	<i>Staphylococcus aureus</i>	19.333	0.577	(18.180, 20.487)
F9	<i>E. coli</i>	21.667	0.577	(20.514, 22.079)
	<i>S. aureus</i>	15.667	1.155	(14.180, 16.487)
Standard (ciprofloxacin)	<i>E. coli</i>	36.333	1.155	(35.254, 37.079)
	<i>S. aureus</i>	35.667	0.577	(34.254, 36.079)

TABLE 9 Mean $\pm$ SEM (mm) inhibitory zone diameter.

Formulations	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>
F8	19.0 $\pm$ 0.33	24.33 $\pm$ 0.33
F9	15 $\pm$ 0.66	22.2 $\pm$ 0.66
<i>Carthamus tinctorius</i> L. oil	18.33 $\pm$ 0.33	20.60 $\pm$ 0.33
Standard (ciprofloxacin)	36.66 $\pm$ 0.66	36.66 $\pm$ 0.66

The comparative analysis of the results in Table 12 provides the binding affinities and interactions of 10 ligands (1_KA to 10_KA) with protein 4HI0. Among the ligands, 3_KA (Figure 13) exhibits the highest binding affinity of -8.3 kcal/mol, indicating a strong interaction with the protein. This ligand forms hydrogen bonds with GLY11, LYS14, THR15, ASP37, and VAL10. It also engages in hydrophobic interactions with ALA16, LYS146, and ARG179. Additionally, electrostatic interactions were observed with LYS146 and ARG179. The presence of multiple hydrogen bonds, hydrophobic contacts, and electrostatic interactions likely contributes to the strong binding affinity of 3_KA. Ligand 2_KA (Figure 14) also demonstrates a high binding affinity of -8.0 kcal/mol. It engages in hydrogen bonding interactions with ASN145, ARG179, ASP148, GLY13, and LYS146. Hydrophobic interactions are observed with THR15, LEU149, and ALA16. This ligand also exhibits electrostatic interactions with ALA16. These interactions contribute to the favorable binding affinity observed. Ligand 1_KA (Figure 15) shows a binding affinity of -7.7 kcal/mol. It forms a hydrogen bond with ASN177 and engages in hydrophobic interactions with LYS146, ARG179, and ALA16. Electrostatic interactions are observed with LYS146. These interactions collectively contribute to the moderate binding affinity of 1_KA. Ligands 9_KA and 7_KA exhibit binding affinities of -7.2 and -6.5 kcal/mol, respectively. Ligand 9_KA forms hydrogen bonds with GLY11, GLY13, LYS14, THR15, and ASP37 while engaging in a hydrophobic interaction with PHE46. Ligand 7_KA forms a hydrogen bond with THR15 and interacts hydrophobically with LYS146 and ARG179. Ligands 10_KA, 8_KA, 6_KA, 4_KA, and 5_KA demonstrate lower binding affinities, ranging from -6.1 kcal/mol to -4.2 kcal/mol. These ligands may have fewer or weaker interactions with the protein. Ligand 10_KA forms hydrogen bonds with THR15, ASP43, and ASP145 while engaging in hydrophobic interactions with LYS146 and ARG179. Ligand 8_KA exhibits an interaction with LYS165, while ligand 6_KA forms hydrogen bonds

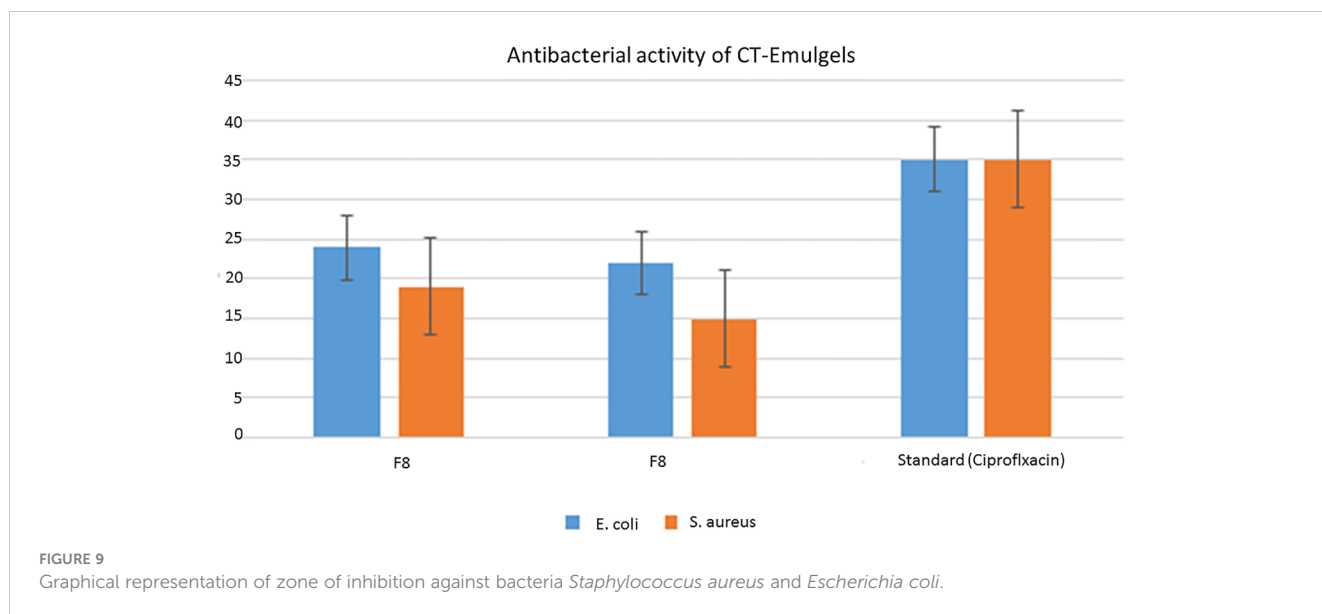


TABLE 10 *Escherichia coli* (n = 3) and *Staphylococcus aureus* (n = 3) MIC and MBC values of pure oil extract.

MIC conc. $\mu\text{g}/100\ \mu\text{l}$ Mean $\pm$ SEM	MBC conc. $\mu\text{g}/100\ \mu\text{l}$ Mean $\pm$ SEM	Bacterial strain tested
0.03 $\pm$ 0.003	0.06 $\pm$ 0.008	<i>E. coli</i>
0.025 $\pm$ 0.003	0.05 $\pm$ 0.075	<i>S. aureus</i>

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

with ILE178, ASN177, and ASP148. Ligands 4_KA and 5_KA show minimal interactions, with only hydrophobic contacts observed.

The comparative analysis of Tables 11, 12 reveals variations in binding affinities and interactions among different ligands with the respective proteins, highlighting the importance of specific molecular interactions in determining ligand–protein binding. Ligands in Table 12 demonstrate a range of binding affinities, with 3_KA and 2_KA exhibiting the highest affinities, emphasizing the significance of hydrogen bonding, hydrophobic contacts, and electrostatic interactions in driving strong ligand–protein interactions. The comparative analysis underscores the diverse nature of ligand–protein interactions, with ligands showing varying degrees of hydrogen bonding, hydrophobic interactions, and electrostatic interactions, providing insights into the molecular mechanisms underlying ligand binding and potential implications for drug discovery and design.

5 Discussion

The optimized CT emulgels were evaluated for *in vitro* antibacterial activity. The disc diffusion method was used for this purpose. The diameter and zone of inhibition (mm) were measured and compared against those of bacterial strains *E. coli* and *S. aureus* as shown in Table 10 and Figure 8. CT oil and optimized emulgels F8 and F9 show good antibacterial activity against both bacteria as documented in the previously published articles, which show that antibacterial activity in CT oil is due to the presence of its active components, which shows a definite mechanism that contributed to its antibacterial activity (Khémiri et al., 2020). Hiba S. et al. concluded that *Myrtus communis*, used as an essential oil, correlated to their active constituents. Wazir et al. showed that the results of antibacterial activity were also due to the occurrence of tannins and flavonoids (Ermawati et al., 2020).

The findings of a one-way ANOVA test revealed a significant value of 0.00, which indicates a probability of less than 0.05 ($p < 0.05$). All of the tested bacteria had a p -value of 0.00, indicating that all the formulations had an antibacterial activity that was statistically significant (Ermawati et al., 2020).

CT oil showed maximum effectiveness against *E. coli* and *S. aureus*. The MIC and MBC values of pure oil were determined by serial dilution technique, which showed significant results. Similar findings have been reported by previous articles in which Wazir A.

TABLE 11 1R4U protein ligands' binding affinity (kcal/mol) and active site binding pocket interactions in angstrom.

Ligands with target protein 1R4U	Isolated extract compounds	Binding score in kcal/mol	Binding pocket interactions of active site
1_KA	Acacetin	−7.1	ARG176 (4.30), ARG176 (4.28), ARG176 (2.37), ASN254 (2.48), GLU259 (3.60), ARG176 (3.79), HIS256 (3.28)
2_KA	Acacetin-7- <i>O</i> - α -L-rhamnopyranoside	−7.4	LEU170 (4.65), TRP174 (4.57), GLU259 (3.66), ARG176 (3.69), HIS256 (3.48), TYR257 (3.22), GLU259 (3.60), THR173 (2.61)
3_KA	Acacetin-7- <i>O</i> - β -D-apiofuranosyl (1 $\rightarrow$ 6)- <i>O</i> - β -D-glucoside	−8.3	LEU170 (3.54), PHE258 (5.35), THR173 (2.30), GLU259 (2.20), PHE258 (5.52), LEU170 (4.79)
4_KA	3Z,5E-Trideca-1,3,5-triene-7,9,11-triyn	−4.3	ARG176 (4.92)
5_KA	3E,11E-Trideca-1,3,11-triene-5,7,9-triyn	−4.4	PHE159 (4.28), VAL227 (4.33),
6_KA	<i>trans</i> -3-Tridecene-5,7,9,11-tetraene-1,2-diol	−4.9	ILE177 (3.00), TYR257 (2.18),
7_KA	2Z-Decaene-4,6-diyne-1- <i>O</i> - β -D-glucopyranoside	−5.8	ARG176 (2.37), TYR257 (2.74), ILE177 (5.06), PHE278 (4.68), ARG176 (2.33)
8_KA	8Z-Decaene-4,6-diyne-1-ol-1- <i>O</i> - β -D-glucuronyl-(1'',2'')- β -D-glucopyranoside	−4.4	PHE159 (4.49), ILE288 (4.92)
9_KA	8Z-Decaene-4,6-diyne-1-ol-1- <i>O</i> - β -D-glucuronyl-(1'',2'')- β -D-glucopyranoside	−6.6	ILE177 (2.08), HIS256 (3.19), THR173 (2.53), TYR257 (2.39), HIS256 (3.74), VAL227 (4.35), ASP175 (2.95), PHE159 (4.06)
10_KA	(2E)-Tetradecaene-4,6-diyne-1,10,14-triol-1- <i>O</i> - β -D-glucopyranoside	−5.8	GLU259 (2.62), GLY286 (3.59), THR173 (2.40), GLN228 (2.40), PHE159 (3.90), HIS256 (2.55)

TABLE 12 Binding affinity (kcal/mol), hydrogen binding, and hydrophobic and electrostatic interactions in angstrom for 4HI0 protein ligands.

Ligands with target protein 4HI0	Isolated extract compounds	Binding score in kcal/mol	Binding pocket interactions of active site
1_KA	Acacetin	-7.7	ALA16 (4.45) LYS146 (4.22), ASN177 (2.61) LYS146 (3.66), ARG179 (4.39), LYS146 (4.89), LYS146 (3.87)
2_KA	Acacetin-7-O- α -L-rhamnopyranoside	-8.0	GLY13 (3.74), LYS146 (3.64), LYS146 (3.87), LEU149 (4.95), ALA16 (4.07), ALA16 (4.43), ASN145 (2.55), ARG179 (2.19), ASP148 (2.53), THR15 (3.70)
3_KA	Acacetin-7-O- β -D-apiofuranosyl (1 $\rightarrow$ 6)-O- β -D-glucoside	-8.3	ALA16 (3.60), LYS146 (4.37), ARG179 (4.87) LYS146 (4.62), ARG179 (4.15), GLY11 (2.39), LYS14 (2.85), LYS14 (2.89), THR15 (2.61), ASP37 (2.75), VAL10 (3.28) ALA16 (3.85)
4_KA	3Z,5E-Trideca-1,3,5-triene-7,9,11-triyn	-4.4	PHE173 (4.75), PRO172 (5.05)
5_KA	3E,11E-Trideca-1,3,11-triene-5,7,9-triyn	-4.2	PHE175 (3.79), LEU157 (4.24)
6_KA	trans-3-Tridecene-5,7,9,11-tetrayne-1,2-diol	-4.9	ASP148 (2.43), ASN177 (2.20), ILE178 (2.45), ASN177 (1.97)
7_KA	2Z-Decaene-4,6-diyne-1-O- β -D-glucopyranoside	-6.5	LYS146 (4.12), ARG179 (4.31), THR15 (2.69)
8_KA	8Z-Decaene-4,6-diyne-1-ol-1-O- β -D-glucuronyl-(1'',2'')- β -D-glucopyranoside	-4.7	LYS165 (4.78)
9_KA	8Z-Decaene-4,6-diyne-1-ol-1-O- β -D-glucuronyl-(1'',2'')- β -D-glucopyranoside	-7.2	ASP37 (2.19), ASP37 (3.28) PHE46 (5.01), GLY11 (2.66), GLY13 (2.16), LYS14 (2.47), THR15 (2.75)
10_KA	(2E)-Tetradecaene-4,6-diyne-1,10,14-triol-1-O- β -D-glucopyranoside	-6.1	LYS146 (3.47), ARG179 (3.33), THR15 (2.62), ASP43 (2.71), ASP145 (2.04)

et al. documented that different methanolic extracts like *Zanthoxylum armatum*, *Terminalia bellirica*, and *Swertia chirata* show the maximum zone of inhibition against both gram-positive and gram-negative strains (Wazir et al., 2014).

According to the present research, the zone of inhibition of CT oil was 18 mm against *S. aureus* and 20 mm against *E. coli*. However, CT emulgel F8 showed a zone of inhibition of 19 mm against *S. aureus* and 24 mm against *E. coli*, and CT emulgel F9

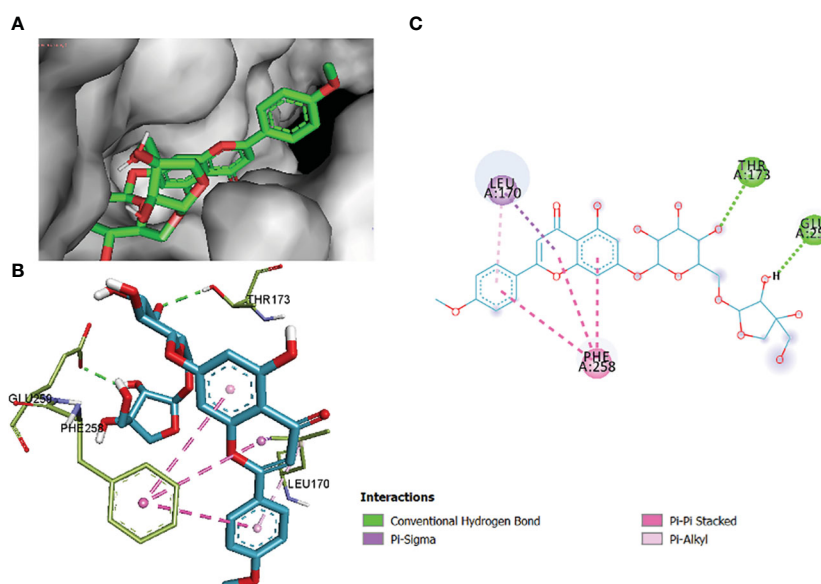


FIGURE 10 3_KA to 1R4U binding modes. Green dashes show H-bond, while other colors show hydrophobic and electrostatic interactions: (A) active site pocket, (B) amino acid interaction, and (C) 2D molecular interactions.

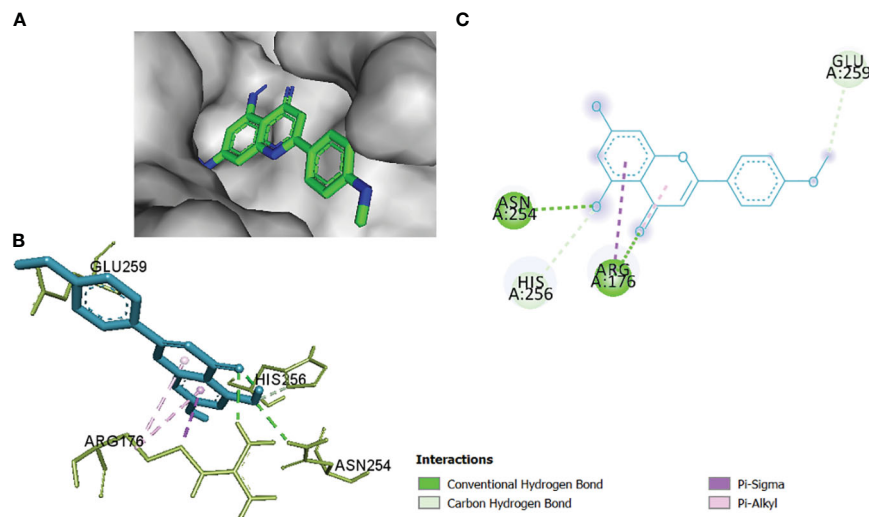


FIGURE 11

1_KA to 1R4U binding modes. Green dashes show H-bond, while other colors show hydrophobic and electrostatic interactions: (A) active site pocket, (B) amino acid interaction, and (C) 2D molecular interactions.

showed a zone of inhibition of 15 mm against *S. aureus* and 22 mm against *E. coli*.

The antioxidant activity of optimized CT emulgels was also checked using DPPH free radical scavenging assay. In this assay, the protons were accepted as free radicals from the antioxidant substrate, decreasing its absorbance, which was reserved as an amount of radical scavenging. Therefore, antioxidant activity was measured by DPPH assay as previously reported by Wazir et al. (2014).

Sample F8 had % inhibition of 42.131 ± 0.335 to 72.440 ± 0.335 at different concentrations. Sample F9 had % inhibition of $26.312 \pm$

0.280 to 42.762 ± 0.398 at concentrations in which both samples F8 and F9 have significant antioxidant potential.

The CT oil was richer in flavonoids and phenolic acids, and antibacterial activity was due to the presence of these components as documented in a previously reported article (Kozłowska et al., 2022).

The previously reported articles concluded that the presence of endogenous free radicals causes oxidative damage, which was protected by the presence of flavonoids and phenolic acids (Chandra and Saklani, 2017; Kozłowska et al., 2022). Kozowska M. et al. also reported that the results of antibacterial and

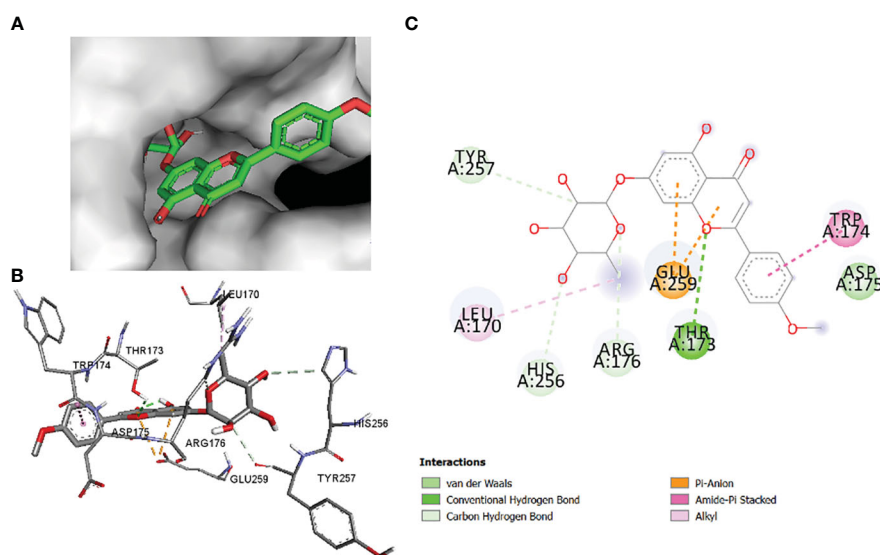


FIGURE 12

2_KA to 1R4U binding modes. Green dashes show H-bond, while other colors show hydrophobic and electrostatic interactions: (A) active site pocket, (B) amino acid interaction, and (C) 2D molecular interactions.

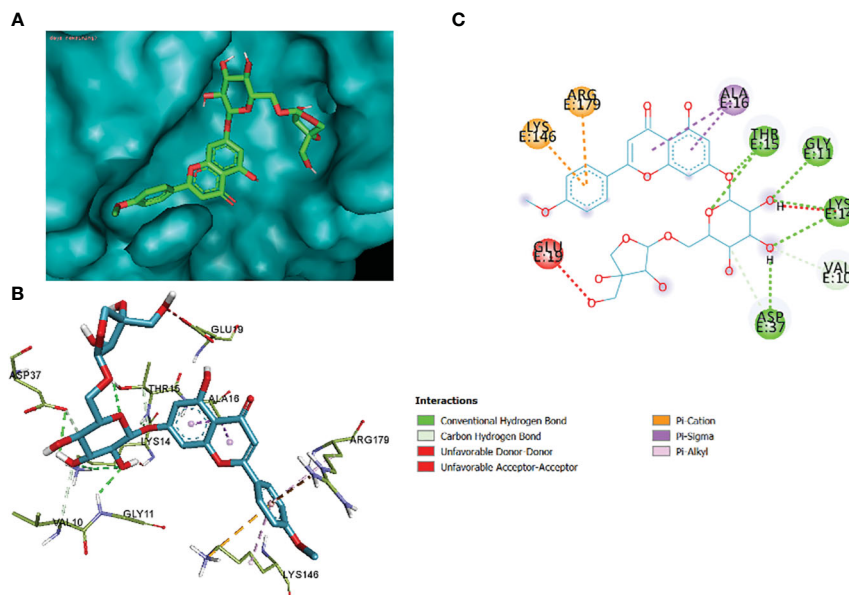


FIGURE 13

3_KA to 4H10 binding modes. Green dashes show H-bond, while other colors show hydrophobic and electrostatic interactions: (A) active site pocket, (B) amino acid interaction, and (C) 2D molecular interactions.

antioxidant activities were also correlated with a high amount of phenolic acid (Kozłowska et al., 2022).

6 Conclusion

This study successfully developed optimized herbal emulgel formulations (F8 and F9) containing *C. tinctorius* L. oil. These formulations exhibited excellent physicochemical properties. This research provided compelling evidence of these medicinal plants' antimicrobial and antioxidant properties without any reported side

effects. In addition, the emulgel was effective against *E. coli* and *S. aureus* *in vitro*, making it a potential treatment for bacterial skin infections. The emulgel was also biocompatible with human skin, which is a good sign of its safety for topical use. This study presents a new, cost-effective topical emulgel formulation containing *C. tinctorius* L. oil as a potential alternative for treating bacterial skin infections. The results cover the methods for additional research to fully understand the mechanisms of action of emulgels in treating bacterial infections.

Furthermore, the study conducted a comparative analysis of molecular docking with antibacterial and antioxidant protein

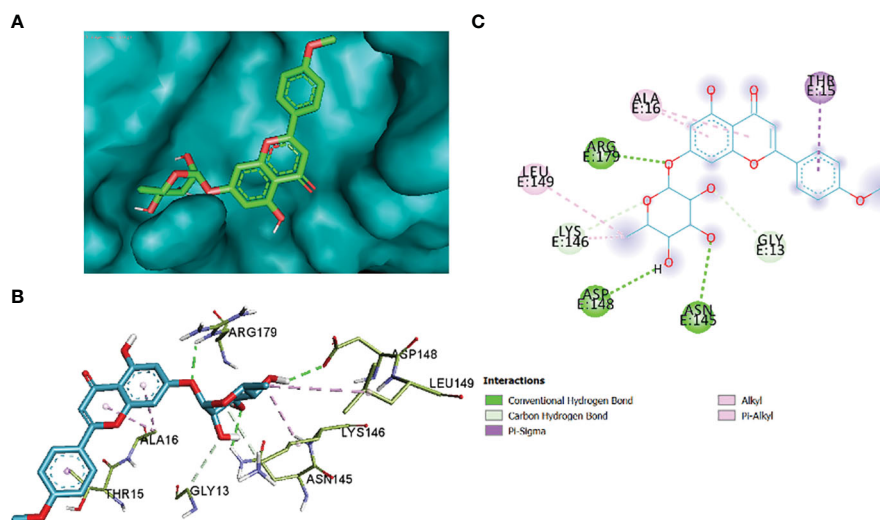


FIGURE 14

2_KA to 4H10 binding modes. Green dashes show H-bond, while other colors show hydrophobic and electrostatic interactions: (A) active site pocket, (B) amino acid interaction, and (C) 2D molecular interactions.

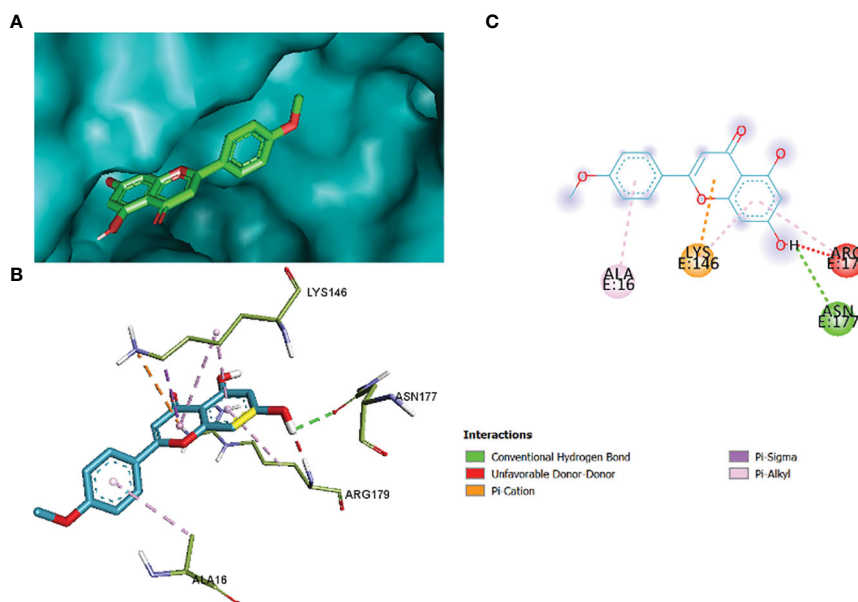


FIGURE 15

1_KA to 4H10 binding modes. Green dashes show H-bond, while other colors show hydrophobic and electrostatic interactions: (A) active site pocket, (B) amino acid interaction, and (C) 2D molecular interactions.

targets. The *in silico* results highlighted the diverse interactions between ligands and proteins, such as hydrogen bonding, hydrophobic interactions, and electrostatic interactions. These findings offer insights into the molecular mechanisms underlying ligand binding, which can have implications for future drug discovery and design. This study will focus on identifying and isolating the pure compounds from the emulgel formulations. This will help determine the individual biological effects of these compounds as antibacterial and antioxidant agents. By doing so, the study aims to contribute to developing new and safer treatment options for bacterial infections and conditions related to oxidative stress. The findings have implications for future drug development and highlight the importance of exploring the therapeutic benefits of medicinal plants in a quest for novel treatments with reduced side effects.

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 current research is approved by the ethical committee of the Faculty of Pharmacy, Bahauddin Zakariya University, under 196/PEC/2022. Informed consent was obtained, and ethical guidelines were followed. The approval letter confirms that all necessary precautions were taken to ensure the protection of human subjects and adherence to ethical standards. Notably, all animal experimentations were conducted in accordance with

applicable laws, regulations, and guidelines, prioritizing animal welfare and minimizing any potential harm.

Author contributions

JS and SS conceptualized the study, performed the experiments, and wrote the manuscript. HJ performed review and editing. AsA, AnA, and TZ performed a formal analysis and refined and improved the manuscript. SS supervised, edited, and conceptualized the study. FS supervised the study, performed *in silico* work, and contributed to manuscript refinement and improvement. GW, YJ, H-AN, and MB performed project administration, review and editing, and data validation. 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.

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Identification of an antivirulence agent targeting the master regulator of virulence genes in *Staphylococcus aureus*

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The emergence of bactericidal antibiotic-resistant strains has increased the demand for alternative therapeutic agents, such as antivirulence agents targeting the virulence regulators of pathogens. *Staphylococcus aureus* exoprotein expression (*sae*) locus, the master regulator of virulence gene expression in multiple drug-resistant *S. aureus*, is a promising therapeutic target. In this study, we screened a small-molecule library using a SaeRS green fluorescent protein (GFP)-reporter that responded to transcription controlled by the *sae* locus. We identified the compound, N-(2-methylcyclohexyl)-11-oxo-10,11-dihydrodibenzo[b,f][1,4]thiazepine-8-carboxamide (SKKUCS), as an efficient repressor of *sae*-regulated GFP activity. SKKUCS inhibited hemolysin production and reduced α -hemolysin-mediated cell lysis. Moreover, SKKUCS substantially reduced the expression levels of various virulence genes controlled by the master regulators, *sae*, and the accessory gene regulator (*agr*), demonstrating its potential as an antivirulence reagent targeting the key virulence regulators. Furthermore, autokinase inhibition assay and molecular docking suggest that SKKUCS inhibits the kinase activity of SaeS and potentially targets the active site of SaeS kinase, possibly inhibiting ATP binding. Next, we evaluated the efficacy and toxicity of SKKUCS *in vivo* using murine models of staphylococcal intraperitoneal and skin infections. Treatment with SKKUCS markedly increased animal survival and significantly decreased the bacterial burden in organs and skin lesion sizes. These findings highlight SKKUCS as a potential antivirulence drug for drug-resistant staphylococcal infections.

KEYWORDS

multiple drug-resistant *Staphylococcus aureus*, antimicrobial resistance, virulence gene expression, Staphylococcal accessory effector, antivirulence agent, SKKUCS

1 Introduction

The emergence of antimicrobial-resistant pathogens is a major challenge for drug development programs and poses a serious risk to public health (Baker et al., 2018; Zhang et al., 2021). *Staphylococcus aureus* is a prominent human pathogen that readily adapts to the host defense mechanisms against infections and causes various life-threatening diseases (Boldock et al., 2018; Kim et al., 2018). The increased incidence of community- and hospital-acquired *S. aureus* over the past decade is due to the rapid emergence of multiple drug-resistant isolates (Al-Mebairik et al., 2016; Rağbetli et al., 2016). Moreover, excessive use of antimicrobials leads to the emergence and spread of resistant strains, significantly compromising their efficacy (Hodille et al., 2017; Troeman et al., 2019). For instance, methicillin-resistant *S. aureus* is endemic to hospitals and community settings and exhibits resistance to various commonly used antibiotics, such as beta-lactams, glycopeptides, and fluoroquinolones. The emergence of vancomycin-resistant *S. aureus* strains has limited the efficacy of conventional antimicrobial treatments against staphylococcal infections, leading to increased morbidity and mortality worldwide (Panlilio et al., 1992; Appelbaum, 2006). Therefore, the development of alternative strategies is necessary to combat *S. aureus* resistance.

Recently, various approaches have been proposed to develop novel drugs for infectious bacterial diseases and improve the treatment efficacy. Of them, the most popular is antivirulence therapy, which interferes with bacterial virulence to prevent bacterial pathogens from invading the host cells and mitigates damage (Arya and Princy, 2013). Various global regulators have been proposed as drug targets because they control the expression of multiple genes involved in staphylococcal virulence functions, such as motility, biofilm formation, toxin production, and immune evasion (Arya et al., 2015; Chemmugil et al., 2019; Kwiecinski et al., 2021). For example, the accessory gene regulator (*agr*) system, which controls the expression of various virulence factors (Sully et al., 2014), has been a popular drug target to attenuate virulence and re-establish susceptibility to antimicrobials in drug-resistant *S. aureus* strains (Gov et al., 2001; Vasquez et al., 2017; Xie et al., 2020). However, the emergence of *agr*-negative clinical isolates in various infections, such as osteomyelitis, mastitis, and cystic fibrosis, limits the widespread application of *agr*-based therapies (Shopsin et al., 2008). To overcome the problems associated with *agr*-based therapeutic approaches, we have been exploring the *S. aureus* exoprotein expression (*sae*) system as an alternative target (Mizar et al., 2018; Yeo et al., 2018).

The *sae* locus encodes the SaeRS two-component signal transduction system composed of SaeP, Q, R, and S. SaeS is a sensor kinase in the cell membrane. Upon recognition of signals such as human neutrophil peptides (HNPs), it autophosphorylates H131 and subsequently transfers the phosphoryl group to SaeR. The phosphorylated SaeR, then, binds to and activates over 20 target promoters, most of which transcribe virulence genes. The *sae* locus has two promoters, P1 and P3. The P3 promoter is constitutive and transcribes only *saeS* and *saeR*, whereas the P1 promoter is autoinduced by Sae and transcribes the entire *saePQRS*.

This locus is involved in the intricate regulation of various virulence genes (Steinhuber et al., 2003; Nygaard et al., 2010) and modulates the production of several key secretory virulence determinants, such as alpha-hemolysin (*hla*), gamma-hemolysin (*hlg*), coagulase (*coa*), Panton-Valentine leukocidin (*lukSF*), and fibronectin-binding proteins (*fnb*) (Steinhuber et al., 2003). As the *sae* locus is an excellent target for developing antivirulence agents, we aimed to investigate it further to facilitate the development of potent antivirulence agents. In this study, we screened a new chemical compound library, Korea Research Institute of Chemical Technology (KRICT), to identify a novel antivirulence compound that can treat staphylococcal infections.

2 Materials and methods

2.1 Ethics statement

All animal experiments adhered to the regulations of the Institutional Animal Care and Use Committee (IACUC). The study protocol was reviewed and approved by IACUC (SKKUIACUC2019-06-13-2), Sungkyunkwan University, School of Medicine. Mice (6–8-week-old) were purchased from Orient Bio Inc. (Seongnam, South Korea) and used for subsequent experiments.

2.2 Bacterial strains and growth conditions

S. aureus strains and plasmids used in this study are listed in [Supplementary Table S1](#). Bacteria were grown in tryptic soy broth (TSB; Sigma-Aldrich, MO, USA). *Escherichia coli* was grown in Lysogeny broth (LB) (Sigma-Aldrich) or agar plates. The reporter strain was maintained in chloramphenicol at 5 µg/mL. The culture was incubated at 37°C with shaking (180 rpm), collected via centrifugation, and washed with sterile phosphate-buffered saline (PBS; Sigma-Aldrich, MO, USA) for injection. The number of viable bacteria was determined after serial dilutions and plating on blood agar ([Supplementary Table S1](#)).

2.3 Construction of GFP-promoter fusion (reporter strain)

Reporter plasmid pCL-P_{hla}min-gfp was generated using a ligation-independent cloning method, as previously described (Donnelly et al., 2006; Yeo et al., 2018). Briefly, pCL55-gfp was used for vector polymerase chain reaction (PCR) with P1969 and P1747 primers (Cho et al., 2015). P1971 and P1972 primers were used to amplify the DNA sequence containing the promoter site. T4 DNA ligase was used to treat the PCR products in the presence of dGTP (insert DNA) or dCTP (vector). Next, *E. coli* DH5α was used for the transformation of the mixed products. After verification, all plasmids were electroporated into *S. aureus* strain RN4220 and subsequently transduced into *S. aureus* USA300 using φ85.

2.4 Screening of the KRICT chemical library

A library of nearly 1,000 chemical products was purchased from KRICT. All compounds were 80% pure; they were dissolved in dimethyl sulfoxide (DMSO; Sigma, USA) and used without any further purification. Drug screening was performed as follows: GFP reporter strain was used for high-throughput screening to identify the antimicrobial and antivirulence potencies of library compounds. GFP reporter strain was collected from TSA plates and suspended in fresh TSB medium. Next, the cultures were incubated at 37°C in a rotary shaker at 180 rpm and allowed to grow to the exponential phase, and cell density was adjusted from 1×10^5 to 5×10^5 CFU/mL. The cultures were added to a 48-well plate (Nunc, Wiesbaden, Germany) with 10 µM of KRICT chemicals and incubated for 8 h. Induced fluorescence of the reporter strain was compared between the vehicle controls and compounds in 0.1% DMSO, and the optical density at 600 nm and fluorescence at 480/520 nm were measured (Biotek, Vermont, USA). Next, the selected lead concentrations were used in a two-fold serial dilution to inhibit the GFP expression by 50% (IC₅₀) and 90% (IC₉₀) compared to that in the vehicle control. The calculated IC₅₀ and IC₉₀ values were used in all subsequent experiments to analyze the efficacy of the selected lead compound.

2.5 Bacterial growth assay

GFP strains were grown overnight in TSB and diluted 1:100 in fresh TSB to reach an early exponential growth phase. Then, 5×10^5 cells were seeded in a 48-well polystyrene plate (Merck, Darmstadt, Germany) without or with effective concentrations of SKKUCS (IC₅₀ and IC₉₀ values of 5 and 10 µM, respectively). The cells were grown at 180 rpm for 10 h at 37°C, and the optical density was measured at 600 nm and compared with that of the vehicle.

2.6 Hemolytic activity assay

Hemolytic activity of SKKUCS was determined with sheep erythrocytes (Icell, South Korea). *S. aureus* USA300 was cultured overnight, diluted to 5×10^5 CFU, and incubated with SKKUCS for 8 hr at 37°C and 180 rpm. The supernatants were collected for further analysis. Then, 5% sheep erythrocytes were prepared by centrifuging 1 mL fresh sheep blood (1620 × g, 10 min) and resuspending the pellet in 1 mL sterile PBS. Culture supernatant with or without SKKUCS (100 µL) were mixed with 100 µL of 5% red blood cells and incubated at 37°C for 30 min. Samples were centrifuged (Eppendorf, Hamburg, Germany) at 1000 rpm, and hemolysis was determined by measuring the optical density of the supernatant at 540 nm with an ELISA plate reader (Biotek, USA) (Tal-Gan et al., 2013; Arya et al., 2015).

2.7 Quantitative reverse transcription-PCR analysis

qRT-PCR was performed for transcripts of interest relative to 16S rRNA using an intercalating dye-based method as previously described, with minor modifications (Mizar et al., 2018). *S. aureus* USA300 cultures were treated with SKKUCS and incubated for 6 h. Total RNA was isolated and purified with the Qiagen RNA Protect Bacteria Reagent and RNeasy Mini Kit (Qiagen; Valencia, CA) via enzymatic disruption. RNA was further purified with silica columns, treated with DNase, and reverse-transcribed into cDNA with a cDNA reverse transcription kit (Random Hexamer; Clontech). qPCR was performed using a CFX Connect Real-Time System (Bio-Rad; Hercules, CA, USA) and SYBR Green PCR Master Mix (Bio-Rad). All samples were assayed in triplicate and normalized to 16S rRNA (Supplementary Table S2) (Park et al., 2007; Jin et al., 2020).

2.8 Cell viability assay

HeLa cells were obtained from the American type culture collection (ATCC; ATCC CCL-2) and propagated in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, California, USA) and 1% penicillin/streptomycin in a 5% CO₂ incubator. Cells were seeded in glass-bottomed dishes (Nunc, USA) at 1×10^6 cells and incubated with SKKUCS (concentration equivalent to IC₅₀ and IC₉₀) for 1 h prior to infection (Stelzner et al., 2021). *S. aureus* USA300 was grown overnight and centrifuged at 4000 rpm for 10 min. The cells were washed with sterile PBS and adjusted to OD₆₀₀ = 1. HeLa cells were infected with USA300 at a multiplicity of infection (MOI) of 1:10 for 45 min. The cells were washed with PBS and then treated with fluorescein diacetate and propidium iodide for 30 min at room temperature in darkness. The cells were washed again with PBS, and fluorescence images were acquired with a confocal microscope (Carl Zeiss Microscopy, Germany) (Wang et al., 2020).

2.9 Invasion assay

RAW264.7 cells (ATCC TIB-71) were used for invasion assays. Briefly, RAW264.7 cells were grown in a high-glucose DMEM medium containing 10% FBS at 37°C with CO₂. When the cells reached 70% confluency, 5×10^5 cells were seeded into a 24-well plate and incubated for 48 h. *S. aureus* USA300 was cultured overnight in TSB and centrifuged at 4000 rpm for 10 min (Zang et al., 2020). The pellet was washed thrice and resuspended in PBS to reach an OD₆₀₀ of 1, corresponding to 10^9 CFU/mL. IC₅₀, IC₉₀, and 2xIC₉₀ doses of SKKUCS were added to the wells 1 h prior to infection. USA300 was applied to RAW264.7 cells at MOI 1:10 and incubated at 37°C with CO₂ for 45 min. The cells were washed, and

gentamicin (100 µg/mL) was added to kill the extracellular or adherent staphylococci. After 60 min incubation, the cells were washed with PBS, and the internalized bacteria were counted by spreading them on blood agar (BAP) plates. The plates were incubated at 37°C for 18 h, and the colonies were counted (Gunaratnam et al., 2019).

2.10 *In vitro* phosphorylation assay

We expressed and purified the cytoplasmic kinase domain of *S. aureus* SaeS (amino acids 118–351) and carried out the *in vitro* phosphorylation assay, as we reported previously (Mizar et al., 2018) with a minor modification. Briefly, purified SaeS (5 µM) was incubated with each test compound (50 µM) or 10% DMSO (vehicle control) in the reaction buffer on ice for 15 min. Then, the mix of ATP and [γ -³²P]ATP (total phosphate donor concentration of 130 µM) was added to initiate the autokinase reaction. The reactions were subjected to SDS-PAGE, and the polyacrylamide gel was further dried on a filter paper and exposed to an X-ray film to visualize the phosphorylated SaeS bands. The phosphorylation levels of SaeS were then quantified by densitometric analysis, and its autokinase activities were calculated as the percentages of phosphorylated protein in the samples challenged with the tested compounds compared to that in the sample challenged with 10% DMSO (vehicle control).

2.11 Molecular docking study

A structural atomic model of the sensor histidine kinase SaeS from *S. aureus* USA300 was collected from an AlphaFold database (Varadi et al., 2022) with an AlphaFoldDB ID of Q2FIT5. The chemical structures of ATP and SKKUCS were obtained from an NCBI PubChem database (Kim et al., 2023) with their PubChem CIDs of 5957 and 4167768, respectively, and were prepared using Open Babel (O'Boyle et al., 2011) prior to the docking study. The *in silico* molecular dockings of ATP and SKKUCS into the kinase domain of *S. aureus* SaeS (residues 118–351) were performed using GNINA (McNutt et al., 2021), which can be accessed via an executable Google Collaboration notebook provided at <https://github.com/gnina/gnina>. To validate the affinity scores derived from GNINA, we estimated the binding affinity of each docking pose of ATP and SKKUCS to SaeS using HAC-Net (Kyro et al., 2023), which can be accessed via an executable Google Collaboration notebook provided at <https://github.com/gregory-kyro/HAC-Net>.

2.12 Animal survival assay after intraperitoneal infection

S. aureus USA300 was grown overnight in TSB. The next day, the culture was diluted 1:100 in fresh TSB medium and allowed to grow to an early exponential growth phase. The culture pellet was washed with DPBS to reach 5×10^7 CFU for the infection. The cells were injected intraperitoneally with 50 µL of DPBS into mice.

DMSO was used as the negative control. Mice were treated with various doses of SKKUCS or vehicle at 12 h post-infection. The mice were administered SKKUCS via the tail vein every 12 h for one week. The mice were monitored daily, and weight loss was recorded. One-week post-infection, the mice were euthanized via CO₂ inhalation, and the kidney, spleen, liver, and heart were excised. The samples were placed in 1 mL of DPBS for homogenization, and the CFU were counted in BAP (Yeo et al., 2018).

2.13 Establishment of the dermonecrosis infection model

For the skin infection experiment, SKKUCS was solubilized at 1 mg/mL in DMSO (Sigma) and diluted further in DPBS for mice injection. DMSO was used as the negative control. BALB/c mice (6–8-week-old, 20–25 g, female) were obtained from Orient Bio Inc (Korea). The lower backs of the mice were shaved, and chemical depilatories were applied. An overnight culture of *S. aureus* USA300 was diluted 100-fold into fresh TSB and further incubated for 4 h at 37°C. The bacterial culture was washed with DPBS and prepared 1×10^7 for infection. The mice were subsequently treated with SKKUCS at 8 h post-infection. The first group of infected mice was subcutaneously treated with 5 µM, the second group with 10 µM, and the third group with 20 µM. The final doses of SKKUCS were 109.9, 219.8, and 439.7 µg/kg, respectively. The chemical was repeatedly administered to the mice for one week at every 12 h interval. The lesion area was measured with calipers as previously described (Cheung et al., 2011; Dokoshi et al., 2020) and recorded daily in addition to body weight loss. The ulcer area (mm²) was calculated using the following equation: $(\pi/2)([\text{length of the abscess}] \times [\text{width of the abscess}])$. After one week, the mice were euthanized via CO₂ inhalation. The ulcer area was excised, and the spleen was removed. The samples were placed in 1 mL DPBS and homogenized for bacterial CFU count. The samples were diluted and plated on blood agar as previously described.

2.14 Statistical analyses

Statistical analyses were conducted using the GraphPad Prism software (version 7.0). Student's *t*-test was used to compare the two datasets. One-way ANOVA analysis of variance was used for more than three datasets. Log-rank test (Mantel–Cox) was used for survival analysis. A non-parametric Mann–Whitney test (two-tailed) was used to compare the two groups. Statistical significance was set at $p < 0.05$.

3 Results

3.1 Small molecule library screening for Sae inhibitor

An overview of the primary screening of antivirulence agents is presented in Figure 1A. The α -hemolysin (*hla*) gene is a well-

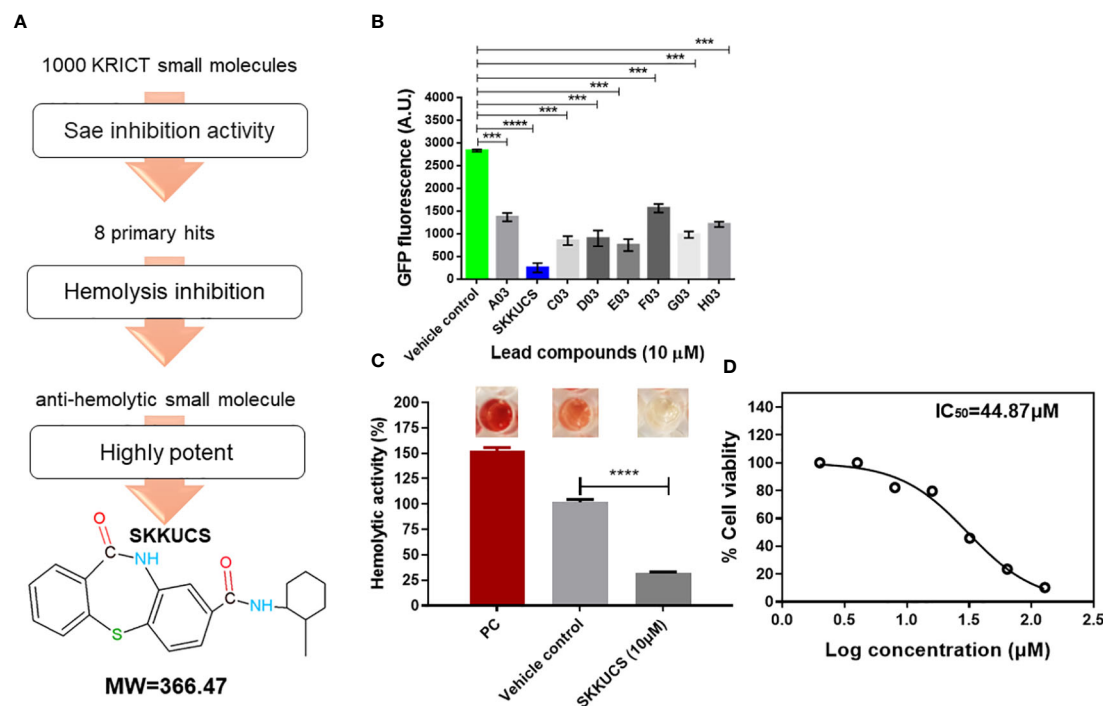


FIGURE 1

N-(2-methylcyclohexyl)-11-oxo-10, 11-dihydrodibenzo[b,f][1,4]thiazepine-8-carboxamide (SKKUCS) suppresses green fluorescent protein (GFP) expression in USA300_{P_{hla}min} strain. (A) Schematic representation of the screening procedure. (B) Potent leads from the Korea Research Institute of Chemical Technology (KRICT) used to reduce GFP expression in USA300-*P_{hla}min*-gfp were analyzed at 8 h. (C) The most potent lead was further tested for hemolysis inhibition and analyzed at 8 h. (D) HeLa cells were seeded, treated with SKKUCS (2–128 μM), and incubated for 72 h. Lactate dehydrogenase (LDH) release was measured at OD_{450nm}. All experiments were performed in triplicate, and significance was compared to the vehicle. Statistical significance was measured using an unpaired two-tailed Student's t-test. ***p < 0.001; ****p < 0.0001.

characterized target of the SaeRS TCS, and its promoter has the SaeR-binding sequence (Nygaard et al., 2010). Therefore, in this study, we decided to screen the KRICT chemical library using a previously constructed SaeRS reporter strain (Mizar et al., 2018; Yeo et al., 2018). We generated a minimal *P_{hla}*, termed *P_{hla}min*, containing only the SaeR-binding site and the -35 and -10 promoter sequences. *P_{hla}min* was fused with the green fluorescent protein (*gfp*) gene in a single-copy integration vector, pCL55, and the resulting plasmid, pCL-*P_{hla}min*-gfp, was inserted into *S. aureus* USA300. The resulting SaeRSGFP reporter strain was used to screen approximately 1000 small molecules from the KRICT library to identify eight compounds that repressed GFP expression by more than 50% at 10 μM. These were considered primary hits (Figure 1B). All primary hits were assessed for their anti-hemolysis activity, and only one molecule exhibited a sharp reduction in hemolysis (Figure 1C). The molecule, N-(2-methylcyclohexyl)-11-oxo-10,11-dihydrodibenzo[b,f][1,4]thiazepine-8-carboxamide (M.W. 366.5), was named SKKUCS and further studied. With USA300-*P_{hla}min*-gfp, SKKUCS showed a half-maximal inhibitory concentration (IC₅₀) of 5 μM and a 90 percent inhibitory concentration (IC₉₀) of 10 μM. At IC₉₀, SKKUCS exhibited a minimal growth inhibitory effect on USA300 (Supplementary Figure S1).

Next, we measured the cytotoxicity of SKKUCS with HeLa cells. using the lactate dehydrogenase (LDH) release assay. HeLa cells were incubated with various concentrations of SKKUCS (2–128

μM), and the release of lactate dehydrogenase (LDH) was measured by monitoring the optical density at 450 nm. SKKUCS showed a mild cytotoxicity to HeLa cells with IC₅₀ of 44.87 μM (Figure 1D).

3.2 Inhibitory effects of SKKUCS on SaeRS GFP reporter and hemolysis

Next, we evaluated the antivirulence effects of SKKUCS. SaeRS GFP reporter was incubated with various concentrations of SKKUCS (5–15 μM), and the fluorescence signal was measured at different time points. As shown in Figure 2A, incubation of GFP-reporter with the IC₅₀ and IC₉₀ concentrations of SKKUCS reduced the *gfp* expression by 48.12 ± 3.9 and 82.4 ± 1.6%, respectively. In addition, incubation with 15 μM SKKUCS for 10 h decreased the fluorescence intensity by 93.03% (Figure 2A). As we aimed to develop a novel SaeRS-based inhibitor specifically targeting the *S. aureus* virulence without affecting its growth, we measured the inhibitory effect of SKKUCS on the growth of *S. aureus* USA300 in a time-dependent manner. SKKUCS showed no significant growth-inhibitory effect up to 10 μM, and showed small growth inhibition at 15 μM (Figure 2B).

SaeR binds to the promoter region of *hla* and activates its expression, which plays a critical role in erythrocyte hemolysis during staphylococcal infections. Here, we tested the effect of SKKUCS on erythrocyte lysis by *S. aureus* USA300. As shown in

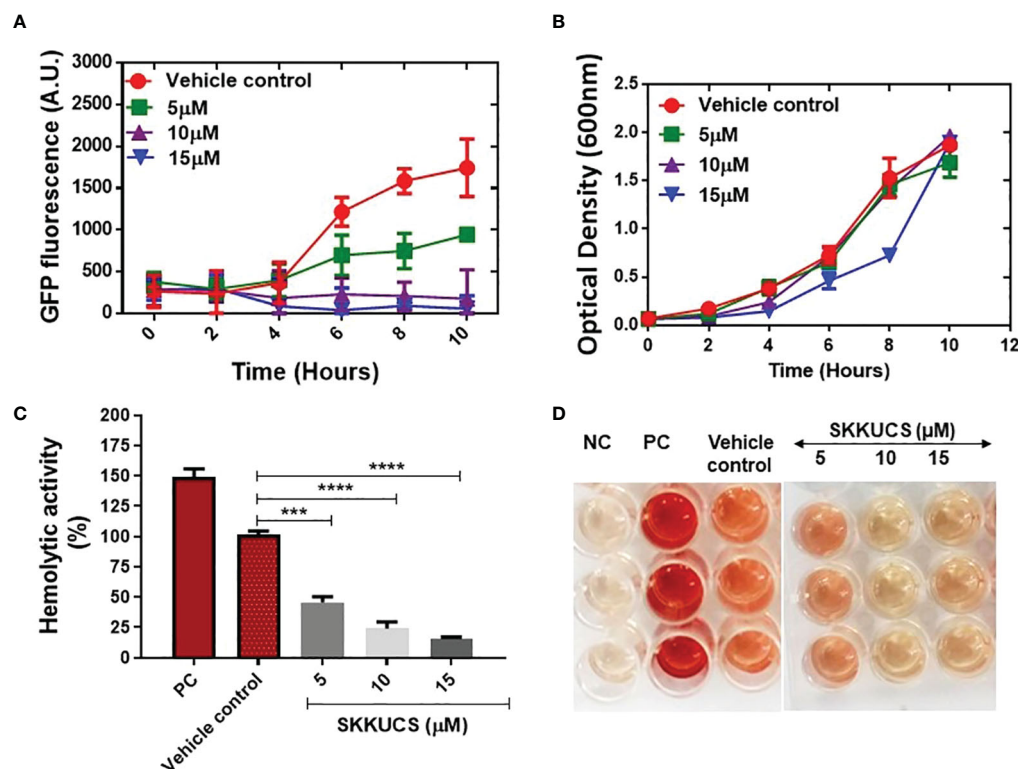


FIGURE 2

Effects of SKKUCS on *Staphylococcus aureus* growth and hemolysis inhibition. (A) USA300-*P_{hlam}-gfp* was grown with or without SKKUCS (5–15 μM) as vehicle control for 10 h. (B) Cell growth was measured in a time-dependent manner with various concentrations of SKKUCS. (C) Various concentrations of SKKUCS were used to treat sheep red blood cells (RBCs), and percentage hemolysis was calculated relative to the vehicle control. (D) Qualitative hemolysis. All experiments were performed in triplicates and significance was compared to the control (no treatment) using one-way analysis of variance (ANOVA). NC, negative control; PC, positive control. *** $p < 0.001$, **** $p < 0.0001$.

Figures 2C, D the IC_{50} and IC_{90} concentrations of SKKUCS reduced the hemolytic activity of USA300 by 45.7 ± 4.7 and $76.0 \pm 5.4\%$, respectively, as compared to the hemolysis observed in non-treated samples. Additionally, hemolysis was inhibited by $85.5 \pm 3.5\%$ when the samples were treated with 15 μM SKKUCS. These results are consistent with the IC_{90} values observed for the SaeRS-GFP reporter strain. Time-dependent assay further demonstrated that SKKUCS had no impact on the growth curve of *S. aureus* USA300 compared to that of the control at various concentrations.

3.3 Effects of SKKUCS on virulence gene expression

We further analyzed the effects of SKKUCS on the transcription of virulence genes. In this analysis, we used 10 μM of SKKUCS, the IC_{90} of the SaeRS GFP reporter. SKKUCS suppressed the transcription of the *sae* operon, except for *saeS* (Figure 3A). Since the SaeRS TCS is required for the expression of *hla* (Gudeta et al., 2019), the suppression of the *sae* transcription explains the inhibitory effect of SKKUCS on the hemolysin activity (Figures 2C, D). Agr is another global regulator required for the *hla* expression. The *agr* locus comprises four regulatory genes, of which *agrA* and *agrC* constitute a two-component system, whereas *agrB* encodes a transmembrane protein required for the processing

of the signal pre-peptide, AgrD. As shown in Figure 3B, SKKUCS suppressed the transcription of *agrA* and *agrB*, raising the possibility that SKKUCS suppresses the Agr system too. To confirm the suppression of the SaeRS TCS, we examine the effects of SKKUCS on the transcription of the Sae regulons. As shown in Figures 3C–G, the transcription of the Sae regulons, except for *coa*, was reduced by SKKUCS, further confirming the Sae-suppressive activity of SKKUCS.

3.4 SKKUCS prevents *S. aureus*-mediated cellular damage

HeLa cells are widely used as a model to explore the cellular responses to pore-forming cytotoxins (Thay et al., 2013). α -hemolysin is well known for its cytotoxicity (Vandenesch et al., 2012; Thay et al., 2013). Therefore, we examined whether SKKUCS can protect HeLa cells from damages induced by α -hemolysin from *S. aureus* USA300. HeLa cells were infected with USA300 in the presence of various concentrations of SKKUCS. Then, the HeLa cells were subjected to Live/Dead staining. Based on the image analysis presented in Figure 4C, it is clear that SKKUCS at IC_{50} concentration has minimal impact on cell viability as compared to the control. In the control group, approximately 22% of HeLa cells infected with USA300 were detected as dead cells and compared

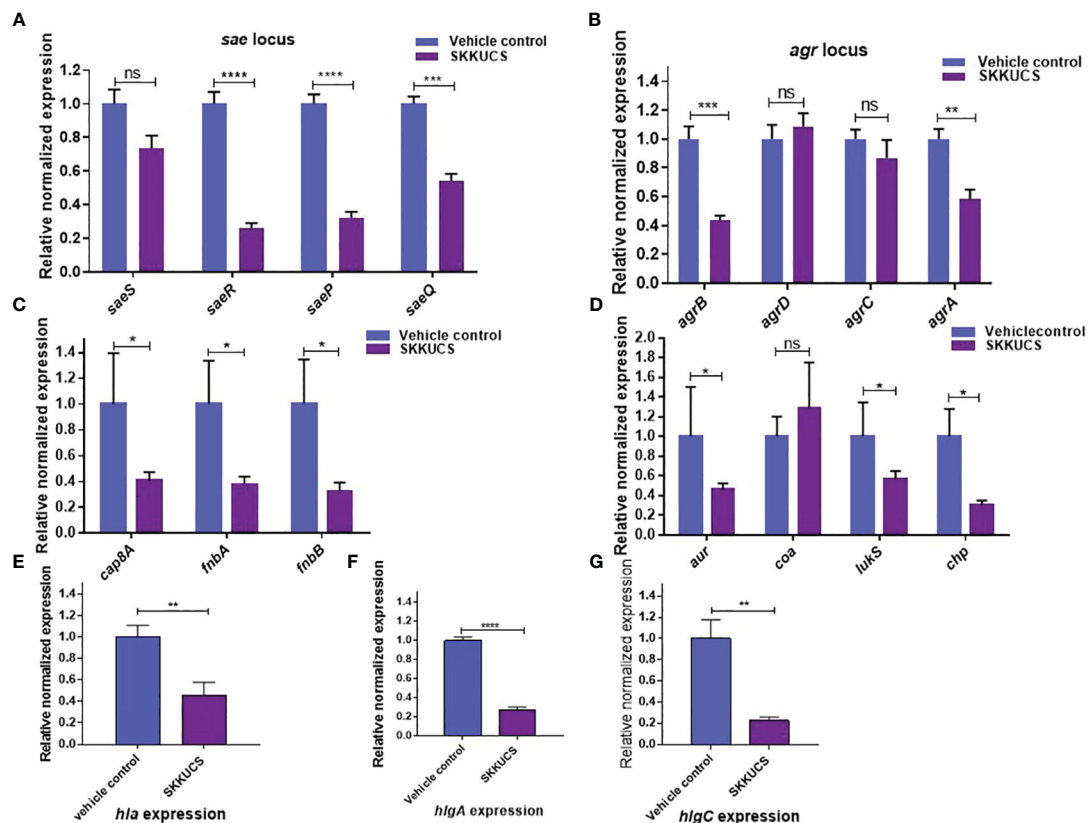


FIGURE 3

Effects of SKKUCS on virulence gene expression. *S. aureus* USA300 was treated with SKKUCS (10 μ M) for 6 h, and the transcription levels of virulence genes were analyzed. (A) Staphylococcal accessory effector (*sae*) locus gene expression. (B) Accessory gene regulator (*agr*) locus gene expression. (C) Genes involved in adherence: capsular polysaccharide (*cap8A*), fibronectin-binding proteins (*fnbA* and *fnbB*). (D) Aureolysin (*aur*), coagulase (*coa*), leukocidins (*lukS*), and chemotaxis inhibitory protein (*chp*). (E) α -hemolysin (*hla*). (F) Y-hemolysin component A (*hlgA*). (G) Y-hemolysin component C (*hlgC*). The Y-axis indicates the mRNA level relative to 16S rRNA, whose transcription was not significantly affected by SKKUCS. All experiments were performed in triplicate, and statistical significance was measured using an unpaired two-tailed Student's t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant.

with no infection control (Figures 4A, B), whereas only 19% of cell death was observed in the IC₅₀-treated group (Figure 4C). However, when USA300 infected HeLa cells were treated with IC₉₀ or 2xIC₉₀, the number of dead cells decreased significantly to 3.5% and 2.6%, respectively (Figures 4D, E). This indicates that SKKUCS was able to protect HeLa cells at these concentrations from damage, in comparison to the vehicle control group (Supplementary Figure S3).

Next, we investigated the effects of SKKUCS on the intracellular survival of USA300 in macrophages (RAW264.7 cells). The SKKUCS treatment at IC₅₀ and IC₉₀ reduced the intracellular survival of USA300 by 41.0 and 78.1%, respectively (Figure 4F). When the concentration was increased to 2xIC₉₀, SKKUCS reduced the intracellular survival of USA300 by 85.91% (Figure 4F).

3.5 SKKUCS inhibits histidine autokinase activity and interacts with SaeS

The kinase activity of SaeS is essential for the virulence of *S. aureus* (Liu et al., 2015). It is possible that SKKUCS exerts its antivirulence activity by interfering with the SaeS kinase activity. To

test the possibility, we examined the effect of SKKUCS and the control compound xi_8141_13 on the autophosphorylation activity of the recombinant SaeS kinase domain (residues 118–351) (Figure 5A). The SaeS phosphorylation was slightly reduced by Xi_8141_13 compared to DMSO; however, it was reduced approximately by 30% by SKKUCS (Figure 5B). To further understand the inhibitory effect of SKKUCS on the autokinase activity of SaeS, we carried out in silico molecular docking experiment. Due to the unavailability of an experimentally resolved structure of *S. aureus* SaeS, we employed an AlphaFold-derived atomic model of the kinase domain of SaeS for the docking study. A structural similarity search using FoldSeek (van Kempen et al., 2023) unveiled a striking resemblance between the C-terminal catalytic and ATP-binding domain of SaeS and those of crystallographically resolved structures of *Thermotoga maritima* HK853 complexed with ADP (Mideros-Mora et al., 2020) and *Bacillus subtilis* WalK complexed with ATP (Celikel et al., 2012), indicating a nucleotide-bound state of the AlphaFold model of SaeS (Figure 5C).

Supporting this observation, molecular docking of ATP into the kinase domain of SaeS revealed that the top-scored docking pose of ATP fitted well to the ATP-binding pocket SaeS (Figure 5D). Remarkably, the conformation of the docked ATP closely

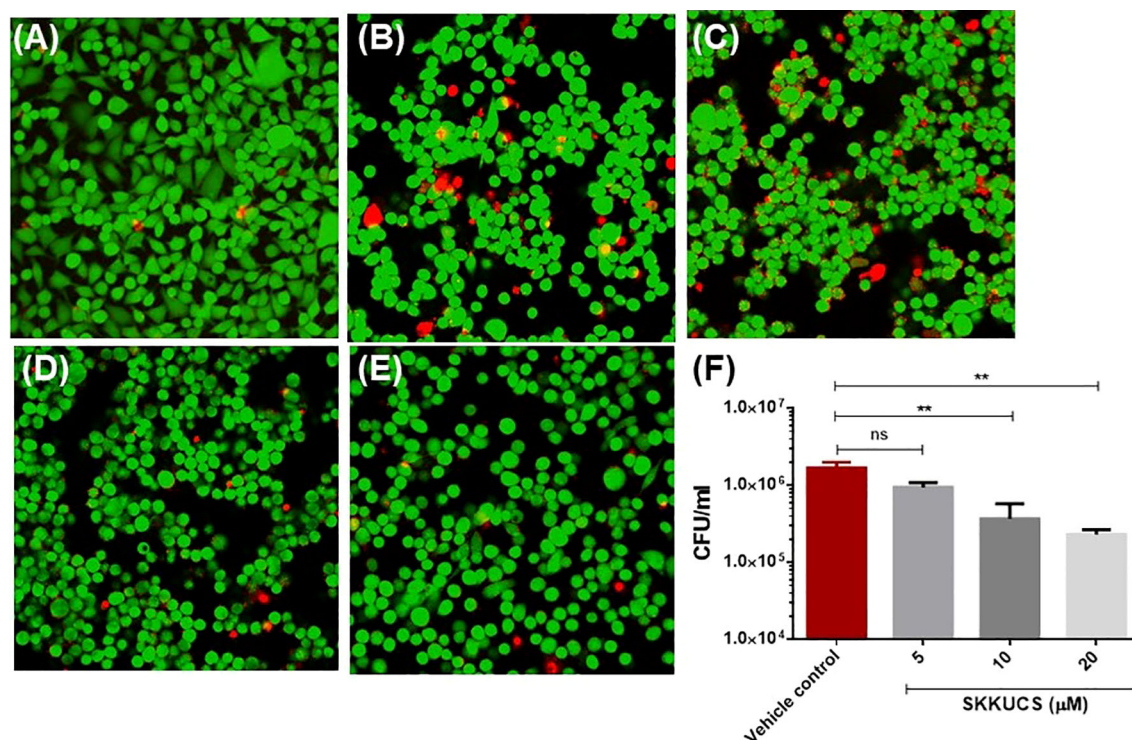


FIGURE 4

SKKUCS protects HeLa cells from α -hemolysin-mediated cell damage and reduces the intracellular survival of *S. aureus* USA300. HeLa cells were seeded in 1×10^6 and treated with SKKUCS. HeLa cells were infected with *S. aureus* USA300 at 1:10 multiplicity of infection (MOI) and incubated for 45 min. Then, cells were stained with fluorescein diacetate (FDA) and propidium iodide (PI) and imaged under the confocal microscope. Green, live; red, dead, (A) no infection; (B) vehicle control; (C) 5 μ M; (D) 10 μ M; (E) 20 μ M. (F) RAW264.7 cells were seeded in 1×10^6 CFU and treated with SKKUCS. RAW264.7 cells were infected with *S. aureus* USA300 at 1:10 MOI and incubated for 45 min. Extracellular *S. aureus* was lysed with gentamicin (100 μ g/mL), and internalized *S. aureus* in blood agar plates was counted. All experiments were performed in triplicate, and statistical significance was measured using an unpaired two-tailed Student's t-test. **p < 0.01; ns, not significant.

resembled those of other nucleotides occupying the similar pockets in WalK and HK853 (Figure 5E). Moreover, SaeS residues implicated in accommodating the docked ATP are similar to those observed in the crystal complex structures of WalK-ATP and HK853-ADP (Figures 5F–H). Collectively, the *in silico* molecular docking study exhibited its efficacy in accurately determining the conformation of ATP within SaeS and thus enabled us to identify the binding site of SKKUCS in the SaeS structure.

The molecular docking of SKKUCS into the kinase domain of SaeS revealed that all three top-scored docking poses of this chemical not only accommodated well to the ATP-binding pocket but also superimposed well to the ATP molecule (Supplementary Figure S2). Consistently observed in these docking poses, the SKKUCS's dibenzothiazepine moieties likely competed with the adenine moiety of ATP, while their methylcyclohexane moieties likely competed with the ATP's phosphate groups. Due to the predominance of ring structures in SKKUCS, it is conceivable that hydrophobic interactions primarily drive the binding of this chemical within the ATP-binding pocket of SaeS (Supplementary Figure S2). In this pocket, SKKUCS probably shared certain interacting residues with ATP, most notably Phe253 and Arg298 (Figure 5H; Supplementary Figure S2).

3.6 Validation of the antivirulence activity of SKKUCS using the mouse peritoneal infection model

Next, we further validated the antivirulence activity of SKKUCS in a mouse model. The mice were challenged with 5×10^7 colony forming units (CFUs) *S. aureus* USA300 via an intraperitoneal (i.p.) injection and treated with SKKUCS at IC₅₀ and IC₉₀ doses (109.9 and 219.8 μ g/kg body weight, respectively) via the tail vein 8 hrs post-infection. Drugs were administered to the mice every 12 hrs for one week. Compared to the no-drug control, IC₅₀ and IC₉₀ doses of SKKUCS protected the mice and increased their survival by 40 and 60%, respectively (Figure 6A). To assess the efficacy of SKKUCS at higher doses, we increased the doses of SKKUCS from 329.8 to 879.5 μ g/kg body weight. SKKUCS administered at a dosage of 329.8 μ g/kg demonstrated comparable efficacy as IC₉₀ and protected the mice up to 60% when compared to the disease control group. High doses of 439.7 and 879.5 μ g/kg SKKUCS significantly increased the survival of animals to 80 and 100%, respectively, compared to that in the disease control. To further investigate the potency of SKKUCS, we examined the bacterial load in a murine intraperitoneal infection model one-week post-infection with USA300. According to the data presented in

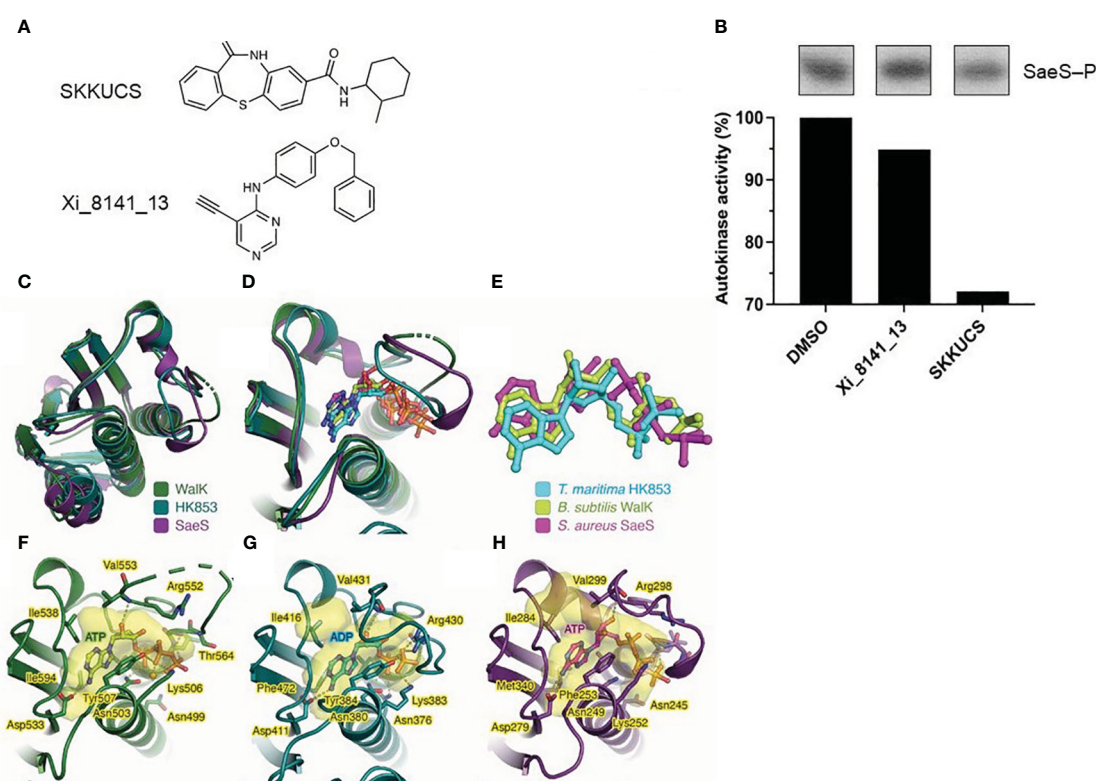


FIGURE 5

Inhibitory effect of SKKUCS on the autokinase activity of SaeS and molecular docking. (A) Chemical structures of compounds tested for their inhibitory effects on the *in vitro* autokinase of SaeS. (B) Autophosphorylations of SaeS in the presence of different tested compounds. Purified recombinant SaeS (5 μ M) was incubated with either SKKUCS or Xi_8141_13 (50 μ M each) for 15 min prior to the addition of ATP (130 μ M) to initiate the autophosphorylation reactions. Following SDS-PAGE analysis, phosphorylated SaeS were visualized by autoradiography and quantified by densitometric analysis. The autokinase activities were calculated as the percentages of phosphorylated proteins in the samples challenged with the tested compounds compared to that in the sample challenged with 10% DMSO (vehicle control). (C) Structural superimposition of the ATP-binding domains of *S. aureus* SaeS (AlphaFoldDB: Q2FIT5), *B. subtilis* Walk (PDB: 3SL2), and *T. maritima* HK853 (PDB: 6RGZ). (D) Structural alignment as in (C), focused on the ATP-binding sites with the bound nucleotides. (E) Top-scored docking pose of ATP within SaeS in superimposition with experimentally determined ADP bound to HK853 and ATP bound to Walk. (F–H) Protein residues interacting with nucleotides observed in (F) *B. subtilis* Walk, (E) *T. maritima* HK853, and (G) *S. aureus* SaeS. Each protein structure is shown as a cartoon representation. Protein cavities accommodating the nucleotide substrate were calculated using PyVOL (Smith et al., 2019) and are represented as transparent surfaces. The bound nucleotide in each structure and the protein residues involved in nucleotide interactions are shown as stick models. Hydrogen and ionic bonds are drawn as yellow dashed lines connecting interacting atoms. Figures were prepared using PyMOL (<http://www.pymol.org>).

Figure 6B, the bacterial burden in the kidney decreased progressively in response to the increasing doses. IC₉₀ dose of SKKUCS reduced the *S. aureus* USA300 burden to 45.6% (3.8×10^5 CFU/mL), and its higher dose (879.5 μ g/kg) reduced the bacterial count by 77.0% (1.6×10^5 CFU/mL). Next, bacterial burdens in the spleen, liver, and heart were calculated and compared with those in the no-drug control. SKKUCS significantly reduced the bacterial counts in a dose-dependent manner. IC₉₀ dose of SKKUCS reduced the bacterial count to 56.5% (1.3×10^5 CFU/mL) in the spleen (Figure 6C), 54.1% (1.7×10^5 CFU/mL) in the liver (Figure 6D), and 39.7% (9.4×10^3 CFU/mL) in the heart (Figure 6E). A higher dose of SKKUCS (879.5 μ g/kg) significantly reduced the bacterial burden in the spleen, liver, and heart by 94.2% (1.3×10^4 CFU/mL), 74.3% (9.6×10^4 CFU/mL), and 78.37% (3.3×10^3 CFU/mL), respectively, compared to that in the disease control.

3.7 SKKUCS treatment *in vivo* attenuates tissue injury

S. aureus is a common cause of skin and soft tissue infection (Moran et al., 2006). Therefore, we further investigated the anti-virulence activity of SKKUCS in an *in vivo* skin infection model. Mice were challenged with *S. aureus* USA300 (1×10^7 CFU) via an intradermal route. At 8 h post-infection, the mice were treated with 109.9 μ g (IC₅₀), 219.8 μ g (IC₉₀), or 439.7 μ g/kg (2xIC₉₀) body weight. SKKUCS was intradermally administered to the mice every 12 h for one week. Mice infected with USA300 without any treatment (vehicle) developed skin ulcers within two days, reaching a maximum area of 144.0 ± 31.6 mm². During the same period, each group treated with the IC₅₀ dose of SKKUCS had considerably reduced lesion size, reaching a maximum of $58.8 \pm$

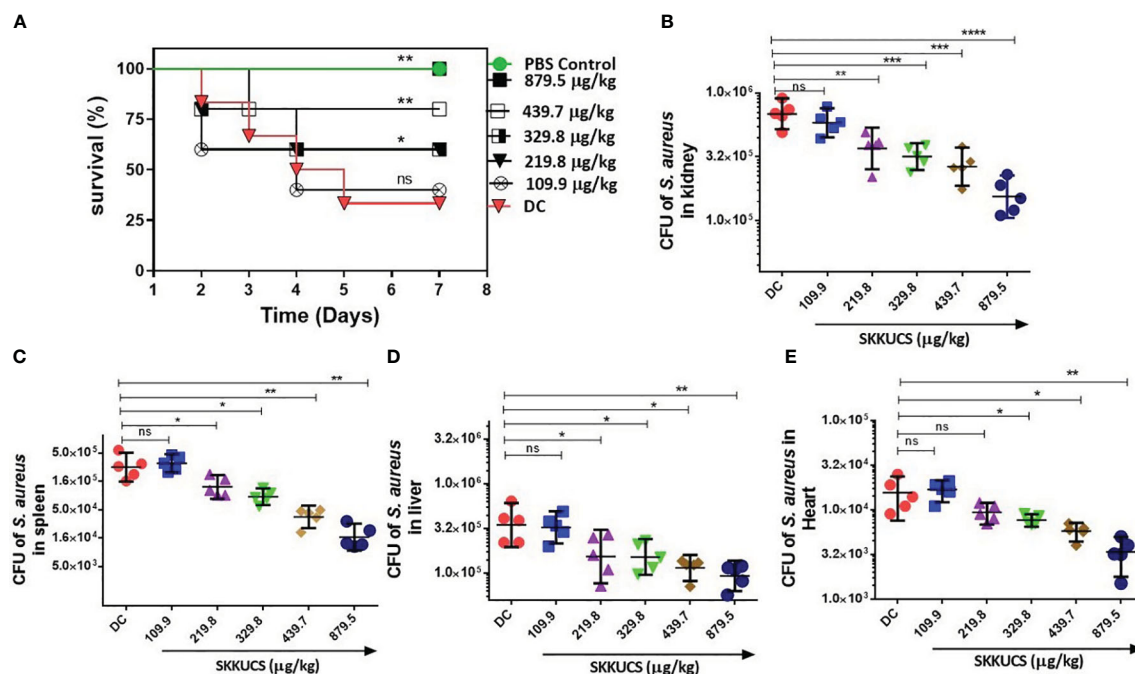


FIGURE 6

Dose-dependent efficacy of SKKUCS in a peritoneal infection model. *S. aureus* USA300 (5×10^7 CFU) was intraperitoneally (i.p.) injected into mice. At 8 h post-infection, SKKUCS (109.9, 219.8, 329.8, 439.7, and 879.5 $\mu\text{g/kg}$ body weight) was injected into the mice via the tail vein. The doses were provided at 12 h intervals for seven days. (A) Seven days post-infection, the percentage survival was calculated as compared to the vehicle. (B) Bacterial burden in the kidney at day 7. (C) Bacterial burden in the spleen at day 7. (D) Bacterial burden in the liver at day 7. (E) Bacterial burden in the heart on day 7. All experiments were performed using biological replicate samples ($n=5$). Statistical significance was calculated using the Log-rank test. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ and ns, not significant via the Student's t-test.

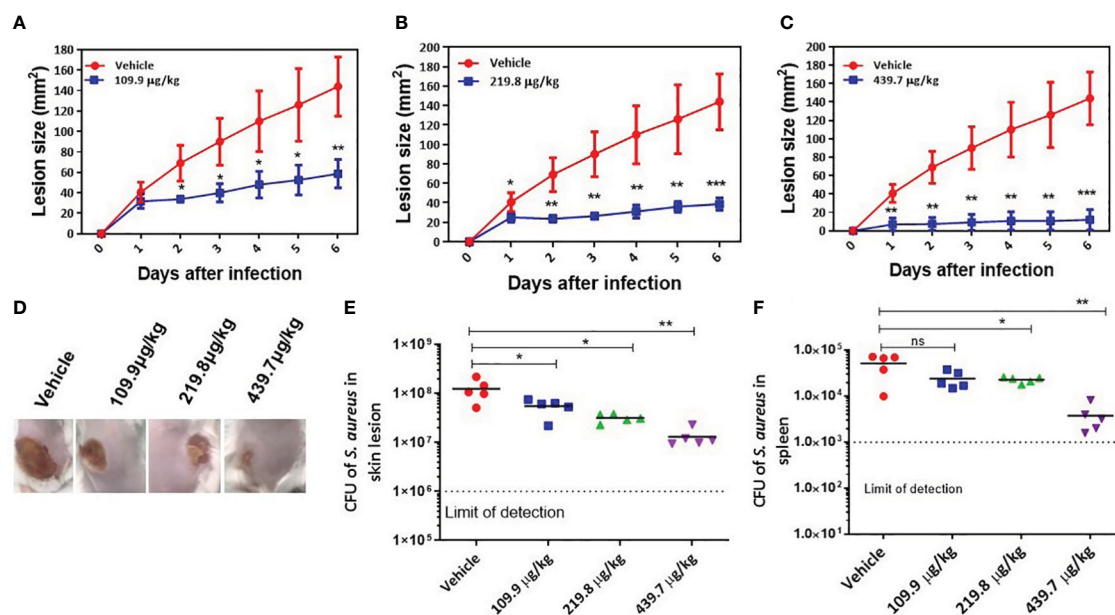


FIGURE 7

In vivo efficacy of SKKUCS in attenuating the dermonecrosis infection. *S. aureus* USA300 (1×10^7 CFU) was injected subcutaneously into the flank ($n=5$ mice per group) of BALB/c mice. The mice were treated with SKKUCS (109.9, 219.8, and 439.7 $\mu\text{g/kg}$ of body weight) or vehicle 12 h post-infection. The mice were treated with SKKUCS at 12 h intervals for one week. Parameters show the lesion development and bacterial burden in the lesion site and spleen. (A) Lesion formation in the group treated with 109.9 $\mu\text{g/kg}$ SKKUCS. (B) Lesion formation in the group treated with 219.8 $\mu\text{g/kg}$ SKKUCS. (C) Lesion formation in the group treated with 439.7 $\mu\text{g/kg}$ SKKUCS. (D) Lesion images were taken one-week post-infection. (E, F) Bacterial burden was calculated in the skin lesion and spleen one-week post-infection. All data from mouse infection were represented as the mean $\pm$ standard error of the mean (SEM) *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ and ns, not significant via the Student's t- and two-tailed Mann-Whitney U tests.

13.8 mm² (Figure 7A). Treatment with the IC₉₀ dose of SKKUCS further reduced the lesion size to 38.8 ± 6.3 mm² (Figure 7B). The 2 × IC₉₀ dose of SKKUCS resulted in an even smaller lesion size of 12.0 ± 11.0 mm² (Figure 7C). As shown in the animal infection site photographs (Figure 7D), SKKUCS inhibited skin lesion formation in a dose-dependent manner. Next, bacterial burden was calculated in the skin and spleen one week after infection. IC₅₀ dose of SKKUCS reduced the bacterial burden in skin lesions by 55.6% (5.4 × 10⁷ CFU/mL), whereas the IC₉₀ dose of SKKUCS reduced the bacterial burden by 74.4% (3.1 × 10⁷ CFU/mL) compared to that in the control. Furthermore, the 2 × IC₉₀ dose considerably reduced the bacterial count in skin lesions by 89.4%, with a bacterial load of 1.3 × 10⁷ CFU/mL (Figure 7E). IC₉₀ dose of SKKUCS reduced the bacterial burden by 55.7% with a bacterial count of 2.2 × 10⁴ CFU/mL, whereas the 2 × IC₉₀ dose of SKKUCS reduced the bacterial burden by 73.2% (1.3 × 10⁴ CFU/mL) in the spleen compared to that in the vehicle-treated animals (Figure 7F).

4 Discussion

The initial screening revealed that SKKUCS markedly inhibited the SaeRS-GFP reporter strain. SKKUCS was the most effective inhibitor of SaeRS-GFP reporter among all the tested small molecules, without any significant reduction in the growth of *S. aureus* USA300. Moreover, analysis of SKKUCS in HeLa cells revealed no significant cytotoxicity, as its IC₅₀ for cell viability was almost four times higher than its calculated IC₉₀ for *S. aureus*. These findings highlight the potential of SKKUCS as a promising lead candidate.

Since *S. aureus* relies on the coordinated expression of several virulence factors for the development and progression of infections, we further investigated the effect of SKKUCS on various prominent virulence factors. Previous studies have suggested that virulence genes such as *aur*, *lukS*, *chp*, *hla*, and γ hemolysin (*hlgA* and *hlgC*) have a direct SaeR-binding site in their promoter regions and are tightly controlled by the SaeRS TCS (Rooijakkers et al., 2006; Cassat et al., 2013; Liu et al., 2016). *S. aureus* relies on the coordinated expression of several virulence factors for the development and progression of infections. Our gene expression analysis revealed that SKKUCS inhibited the expression of genes associated with the *sae* and *agr* loci. Interaction between the *sae* and *agr* loci remains ambiguous. However, previous studies have suggested that these loci function independently (Liu et al., 2016), indicating that SKKUCS potentially interacts with both global regulators. By inhibiting the expression of both *agr* and *sae* regulators, SKKUCS can disrupt the expression of various virulence factors, thereby attenuating the pathogenicity of *S. aureus*. Therefore, targeting multiple loci can aid in the development of potent antivirulence strategies. Furthermore, previous studies showed that interference with global regulators such as *agr* does not exert any selective pressure and, therefore, does not induce resistance against antivirulence agents (Sully et al., 2014).

We further examined the inhibition of pore formation by SKKUCS. The results suggested that SKKUCS may prevent the influx of α -hemolysin and protect the membrane integrity of target cells by inhibiting hemolysin-mediated pore formation in host cell

membranes. Moreover, SKKUCS improved the macrophage function by enhancing the phagocytosis and overall clearance of *S. aureus*. By enhancing macrophage-based clearance, SKKUCS also reduced the bacterial burden and prevented the spread of *S. aureus* infection.

In vivo results revealed that SKKUCS significantly enhanced the clearance of *S. aureus*, probably by promoting immune cell recruitment and reducing the bacterial burden in various organs. Hemolysins aid in the evasion of immune responses and hemolysin-deficient strains are efficiently cleared by immune cells, resulting in decreased *S. aureus* burden, reduced tissue damage, and improved host survival (Haslinger-Löffler et al., 2005; Spaan et al., 2014; Smith et al., 2018). Here, our findings highlight the potency of SKKUCS as an antivirulence agent that can target specific virulence factors. By inhibiting hemolysin production, SKKUCS disrupts the key mechanism used by *S. aureus* to evade host immune responses. Moreover, treatment with the IC₉₀ or an equivalent dose of SKKUCS significantly increases the host survival up to 60%.

Intriguingly, the binding affinities to SaeS of all top-scored docking poses of SKKUCS were estimated to be higher than that of the top-scored docking pose of ATP (Supplementary Table S3). This computational prediction implied that SKKUCS potentially functioned as an ATP competitor in inhibiting the SaeS activity. Additionally, our results from dermonecrosis infection suggest that SKKUCS efficiently interferes with the virulence gene expression of *S. aureus* USA300 *in vivo*, leading to reduced tissue damage and inflammation. Substantial reduction in the bacterial burden in the lesion site further confirmed the efficacy of SKKUCS in inhibiting *S. aureus* transmission. Notably, *S. aureus* count was significantly reduced in the spleen after treatment with SKKUCS during dermonecrosis. These results confirm the efficacy of SKKUCS in limiting the spread of infections and preventing secondary infections associated with systemic dissemination.

In conclusion, SKKUCS acts as a novel potent therapeutic antivirulence candidate and inhibits the expression of *hla* in multidrug-resistant *S. aureus*. The *sae* locus tightly controls the expression of *hla* and serves as a crucial regulatory system for α -hemolysin production in *S. aureus* (Gudeta et al., 2019). Based on the results obtained from autokinase inhibition assay and molecular docking suggest that SKKUCS inhibits the kinase activity of SaeS and potentially targets the active site of SaeS kinase, possibly inhibiting ATP binding. Nonetheless, further research is required to elucidate the precise mechanism. Disruption of the SaeRS system notably decreases the expression of *hla*. Additionally, SKKUCS may interfere with the downstream transcriptional regulators or effectors that are under the direct control of the SaeRS system, leading to a reduction in hemolysin production. Overall, the efficacy of SKKUCS as an antivirulence agent suggests interference with the *sae* locus as a promising approach to control the virulence of *S. aureus*. Previous studies have also suggested that the sensing domain of SaeS from *S. aureus* is highly conserved across strains, and no homologues are found in other bacterial species (Giraud et al., 1999; Flack et al., 2014). Thus, SKKUCS has a narrow-spectrum antivirulence function and no activity towards other pathogens. However, further investigations are necessary to elucidate the specific mechanisms by which SKKUCS exerts its inhibitory effects, facilitating the development of antivirulence agents against *S. aureus* and mitigating the associated clinical challenges.

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 animal study was approved by Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KK: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. RA: Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. JY: Investigation, Writing – original draft. TK: Methodology, Validation, Writing-review & editing, Data curation, Writing - original draft. TB: 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

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The effects of nano-silver loaded zirconium phosphate on antibacterial properties, mechanical properties and biosafety of room temperature curing PMMA materials

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Polymethyl methacrylate (PMMA) frequently features in dental restorative materials due to its favorable properties. However, its surface exhibits a propensity for bacterial colonization, and the material can fracture under masticatory pressure. This study incorporated commercially available RHA-1F-II nano-silver loaded zirconium phosphate (Ag-ZrP) into room-temperature cured PMMA at varying mass fractions. Various methods were employed to characterize Ag-ZrP. Subsequently, an examination of the effects of Ag-ZrP on the antimicrobial properties, biosafety, and mechanical properties of PMMA materials was conducted. The results indicated that the antibacterial rate against *Streptococcus mutans* was enhanced at Ag-ZrP additions of 0%wt, 0.5%wt, 1.0%wt, 1.5%wt, 2.0%wt, 2.5%wt, and 3.0%wt, achieving respective rates of 53.53%, 67.08%, 83.23%, 93.38%, 95.85%, and 98.00%. Similarly, the antibacterial rate against *Escherichia coli* registered at 31.62%, 50.14%, 64.00%, 75.09%, 86.30%, 92.98%. When Ag-ZrP was introduced at amounts ranging from 1.0% to 1.5%, PMMA materials exhibited peak mechanical properties. However, mechanical strength diminished beyond additions of 2.5%wt to 3.0%wt, relative to the 0%wt group, while PMMA demonstrated no notable cytotoxicity below a 3.0%wt dosage. Thus, it is inferred that optimal antimicrobial and mechanical properties of PMMA materials are achieved with nano-Ag-ZrP (RHA-1F-II) additions of 1.5%wt to 2.0%wt, without eliciting cytotoxicity.

KEYWORDS

PMMA, AgNP Ag nanoparticle, mechanical properties, antibacterial properties, biosafety

1 Introduction

Polymethyl methacrylate (PMMA), recognized for its commendable biological, physical, and chemical properties along with superior machinability, has ascended as a predominant material in the field of stomatology (Rao et al., 2022). Employed as a material for denture reconstruction, orthodontic mobile appliances, periodontal splints, and jaw cystoccluders, room-temperature curing PMMA, while boasting numerous advantages, also manifests conspicuous disadvantages. Primarily, the rough surface and porosity of PMMA materials facilitate the colonization of microorganisms, such as *Candida albicans*, *Streptococcus sanguineus*, and *Streptococcus mutans*, thereby propelling the incidence of various oral diseases (An et al., 2022; Moreno-Maldonado et al., 2012; Quassem and Mahross, 2018; Sahm et al., 2022; Pourhajibagher et al., 2023; Schubert et al., 2019; Al-Dwairi et al., 2019; Patil et al., 2020; Dantas et al., 2016). Moreover, PMMA material exhibits a challenge regarding mechanical strength, proving susceptible to breakage under masticatory pressures (Zafar, 2020; Ali Sabri et al., 2021; Alkordy and Alsaadi, 2014; Mohd Farid et al., 2022; Khan et al., 2022; Olewi et al., 2019; Zafar, 2018; Rana et al., 2021). Considering the myriad and multifaceted bacteria present in the human oral cavity, alongside the insufficient physical and mechanical strength of PMMA materials, an imperative arises to enhance the antibacterial properties of PMMA materials without compromising their physical and mechanical attribute.

In recent years, nano-antibacterial materials, notably nano-silver, have garnered considerable attention due to their broad-spectrum and enduring antibacterial effects without inducing bacterial resistance (Y. Liu et al., 2023; Y.-M. Wu et al., 2022). Research indicates that the incorporation of nanoparticles, which contain Ag, into PMMA materials can augment not only the antibacterial prowess of the PMMA materials but also enhance their mechanical properties, thus aptly fulfilling clinical requirements (Bacali et al., 2020; Oyar et al., 2018; Garcia et al., 2021; Chen et al., 2017; Alhotan et al., 2023). Recent studies indicate that incorporating metal nanoparticles, such as silver nanoparticles, into inorganic carriers can enhance nanoparticle dispersion in polymers and mitigate the aggregation of these nanoparticles due to surface effects. This improvement facilitates the optimal utilization of the antibacterial properties of silver nanoparticles (Liu et al., 2019). Common carriers for nano-silver include zinc oxide (Pham et al., 2021), silica (Wong et al., 2017), montmorillonite (Fernández Solarte et al., 2019), zeolite (Youssef et al., 2019), graphene oxide (Huong et al., 2020), and zirconium phosphate (Liu et al., 2022). Researchers have investigated the incorporation of zinc oxide (Mousavi et al., 2020), silicon dioxide (Aati et al., 2022), graphene oxide (Arslan et al., 2023), and zeolite (Malic et al., 2019), all containing nano-silver, into PMMA materials. These studies explore the influence on the antimicrobial and mechanical properties of PMMA, offering new insights for clinical enhancements of these properties. However, zirconium α -phosphate, distinct from the carriers mentioned earlier, exhibits a two-dimensional layered nanosheet structure. It possesses exceptional ion exchange capabilities, catalytic activity,

stable physicochemical properties, and biosafety, making it a versatile carrier for various medicines with significant potential in biomedicine (Saxena et al., 2013; Ramos-Garcés et al., 2021; Nocchetti et al., 2022). Moreover, the layered structure of zirconium phosphate nanosheets allows for increased surface area upon exfoliation, enabling a higher loading capacity for silver nanoparticles and thus enhancing the material's antibacterial efficacy (Nocchetti et al., 2022). Despite these advances, research on the effects of nano-Ag-ZrP in PMMA materials, particularly regarding their antibacterial and mechanical properties, remains limited, suggesting an area ripe for further investigation.

Consequently, this study employs commercially available nano Ag-ZrP (RHA-1F-II) as the research subject, integrating it with room temperature curing PMMA material for material characterization analysis, and examines the impacts of nano Ag-ZrP antibacterial agent on the antibacterial attributes, mechanical properties, and biosafety of the room temperature curing PMMA material.

2 Materials and methods

2.1 Materials

Self-setting denture base polymer (NISSIN, Japan); self-setting denture water (NISSIN, Japan); Nano-Ag-ZrP (RHA-1F-II, China); BHI broth medium (Hopebio, China); LB broth medium (Hopebio, China); Nutritional Agar (Sigma, USA); SYTO 9/PI Live/Dead Bacterial Staining Kit (Thermo Fisher, USA); DMEM culture medium (Gibco, USA); Fetal Bovine Serum (FBS; Gibco, USA); 0.25% trypsin (Gibco, USA); Cell Counting Kit-8 (CCK-8); Calcein-AM/PI Live/Dead Cell Staining Kit (Beyotime, China); Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, China).

2.2 Preparation of specimen

PMMA powder and nano-Ag-ZrP were amalgamated in predefined mass fractions, subsequently subjected to an 8-hour processing in a planetary ball mill to procure the mixed powder. The material of the ball milling tank is zirconia, with a volume of 50 ml and a working volume of 15 ml; The material of the grinding ball is zirconia, with a total mass of 50 g, a diameter of 5 mm, and a ball to material ratio of 5:1; The working speed of the ball mill is 180 rpm/min. The requisite mass fractions of nano-Ag-ZrP for experimentation were defined as 0wt%, 0.5wt%, 1.0wt%, 1.5wt%, 2.0wt%, 2.5wt%, and 3.0wt%. Through combining the mixed powders with denture water, samples of disparate shapes and sizes were fabricated, culminating in the formation of nano-Ag-ZrP/PMMA materials. Conforming to relevant standards (ISO 22196:2007; ISO 20795-1:2008; ISO 527:2012), the antimicrobial specimen dimensions were set at $(50 \pm 2) \text{ mm} \times (50 \pm 2) \text{ mm} \times (2 \pm 0.5) \text{ mm}$; the three-point bending specimen at $64 \text{ mm} \times (10.0 \pm 0.2) \text{ mm} \times (3.3 \pm 0.2) \text{ mm}$; the modified three-point bending specimen at $39 \text{ mm} \times (8.0 \pm 0.2) \text{ mm} \times (4.0 \pm 0.2) \text{ mm}$, featuring a central notch measuring $(3.0 \pm 0.2) \text{ mm}$ in length and 0.5 mm in

width. The tensile test specimen adhered to a 5B dumbbell shape, with an overall length of 35.0 mm, end width of (6.0 ± 0.5) mm, narrow section width of (2.0 ± 0.1) mm, and a thickness of 1.0 mm.

2.3 Nano Ag - ZrP characterization

Surface morphology and elemental mapping of nano-Ag-ZrP were scrutinized utilizing field emission scanning electron microscopy (SEM; FEI, nova nanosem650, America) and transmission electron microscopy (TEM; FEI, F20, America). Additionally, the functional groups, crystal compositions, and valence states of Ag-ZrP were characterized through Fourier transform infrared spectroscopy (FT-IR; Nicolet, iS10, America), X-ray diffraction (XRD; Bruker, D8, Germany), and X-ray photoelectron spectroscopy (XPS; Thermo Fisher, ESCA LAB 350Xi, America).

2.4 The separation, culture and characterization of primary HGFs

Ethical approval was obtained from the Ethics Committee of the Stomatology Hospital of Jilin University. Gingival tissue, procured from patients aged 18–28 undergoing mandibular third molar extraction, necessitated the adjacent mandibular third molar to be healthy. Upon acquiring patient consent, samples were harvested around the extraction wound. Primary Human Gingival Fibroblasts (HGFs) were isolated and cultured utilizing the tissue block method. Optimal efforts were exerted to remove the epithelial tissue utilizing ophthalmic scissors. Gingival tissue, post-epithelial removal, was subjected to 50 repeat washes with DMEM medium, supplemented with 400 U/mL penicillin and 0.4 mg/mL streptomycin, until overt blood stains and impurities were absent. The tissue was then dissected into approximately 1 mm³ tissue blocks using ophthalmic scissors within a 60 mm culture dish. Subsequently, the tissue blocks were uniformly seeded into the base of T25 culture flasks with an approximate spacing of 0.5 cm between each block. Each flask was inoculated with about 10 tissue blocks, complemented by 5 ml of DMEM medium enriched with 20% Fetal Bovine Serum (FBS). The culture flasks were then incubated at 37°C, 5% CO₂ for 4 hours, following which, the flasks were gently inverted and returned to the incubator. Minimal disturbance of the culture flasks was endeavored for the initial 3 days, with medium changes executed every 3 days. Once the primary cells occupied 60–70% of the flask bottom, the first passage was conducted. HGFs from passages 4–6 were designated for experimental use. Characterization of the isolated HGFs was accomplished through immunohistochemistry and immunofluorescence.

2.5 Detection of biosafety

2.5.1 Preparation of specimen extract

The specimen was fashioned into a 10 mm x 10 mm x 2 mm round sheet. Adhering to ISO 10993-12:2012, extracts were

formulated at an extraction ratio of 3.0 cm²/mL. For each group, samples were positioned within centrifuge tubes, accompanied by 733 µl of DMEM culture medium, which contained 10% FBS and 1% dual antibodies. Subsequently, the centrifuge tubes were incubated at 37°C for a duration of 72 hours. Extracts were thereafter collected and reserved for subsequent utilization.

2.5.2 Detection of cytotoxicity by CCK-8

Three 96-well culture plates were seeded with 1.0×10^4 cells per well and incubated at 37° for 24 hours. Subsequently, the culture medium was aspirated, and wells were rinsed with PBS before the addition of 100 µl of extract from each specimen group to the respective wells. On the conclusion of the 1st, 2nd, and 3rd days, a well plate was selected, the spent culture medium was discarded, and 100 µl of CCK-8 culture medium was introduced to each well. Following a 1-hour incubation period, absorbance (OD) values were measured using an enzyme-linked immunosorbent assay apparatus, and the relative cell proliferation rate (RGR) was calculated utilizing the following formula:

$$\text{RGR} = (\text{OD}_{\text{control}} / \text{OD}_{\text{samples}}) \times 100 \%$$

where OD_{samples} represents the absorbance value of each extract group, and OD_{control} denotes the absorbance value of the negative control group.

2.5.3 Live and dead cell staining test

Cells were seeded into 6-well plates at a density of 1.0×10^5 cells per well and incubated for 24h. Subsequently, the culture medium was aspirated, and 2 ml of extract for each group was added for further culturing. After an additional incubation of 72h, the extract was removed from each well, followed by the addition of Calcein-AM/PI staining solution, and a subsequent 30-minute incubation at room temperature, protected from light, ensued. Observations were conducted using a fluorescence microscope.

2.5.4 Annexin V-FITC/PI flow cytometry was used to detect apoptosis

Cells were cultured in 6-well plates at a density of 1.0×10^5 cells per well and incubated for 24h. Following incubation, the culture medium was aspirated and wells were washed with PBS; subsequently, extracts for each group were introduced and maintained for an additional 72h. Cells were then harvested by centrifugation, and Binding Buffer was utilized to resuspend the cells, adjusting the cell concentration to 1.0×10^6 /mL. A 100 µl aliquot of cell suspension was transferred to a centrifuge tube, and Annexin V-FITC and PI dyes were added according to the manufacturer's instructions. The mixture was incubated for 20 min in dark conditions, followed by analysis via flow cytometry.

2.6 Detection of antibacterial property

2.6.1 Preparation of bacterial suspensions

S. mutans (ATCC 700610) and *E. coli* (ATCC 25922) were revitalized. Individual colonies of *S. mutans* and *E. coli* were

isolated and cultured in Brain Heart Infusion (BHI) and Lysogeny Broth (LB) media, respectively, and incubated at 37°. Following an 8-hour incubation period, the optical density (OD) of the bacterial solutions was ascertained using a spectrophotometer. Subsequently, the concentrations of the bacterial suspensions were standardized to 1.0×10^6 CFU/ml to prepare working bacterial suspensions.

2.6.2 The antimicrobial rate of each group was determined by film coating method

Both sides of the specimens were exposed to ultraviolet light for 12 hours, followed by transfer to sterile petri dishes on an ultra-clean workbench. Working bacterial droplets of *S. mutans* and *E. coli*, each measuring 100 µl, were absorbed and introduced to the surface of each specimen group. Polyethylene film was applied to the surface of each specimen group, ensuring close contact with the specimen, and then incubated at 37° for 24 hours. Subsequently, the specimens and films were cleaned with 20 ml of 0.85% NaCl solution, and the collected eluent was vigorously shaken and diluted in a two-fold ratio. The diluted eluent, 100 µl, was plated and incubated at 37°. After 36 hours, colonies were enumerated on the agar plates. The antimicrobial rate was calculated using the formula $R=(B-C)/B$, where R represents the antibacterial rate, B is the average colony count of specimens in the 0%wt group (CFU/ml), and C denotes the average colony count in the experimental group (CFU/ml).

2.6.3 SYTO 9/PI staining of live and dead bacteria

The size of the specimens adhered to the dimensions specified in Section 2.5.1. Specimens from each group were positioned in 48-well plates, with each well receiving 500 µL of a working bacterial suspension of *S. mutans* and *E. coli* for a 24-hour incubation period. Subsequently, the bacterial solution from each well was aspirated and the specimens were rinsed with 0.85% NaCl solution. Specimens from each group were then transferred to centrifuge tubes, combined with 3 ml of 0.85% NaCl solution, and the bacteria from the specimen surfaces were collected following an ultrasonic shock for 5 minutes. Bacterial solutions for each group were centrifuged at 10000 g for 10 minutes; thereafter, the supernatant was discarded, and 200 µL of staining solution was added to suspend the bacterial pellet, followed by incubation in the dark at room temperature for 15 minutes. Finally, 5 µL of the bacterial solution was extracted from each tube, applied to a slide, and observed via a fluorescent inverted microscope.

2.6.4 Bacterial adhesion test

The specimen size and the co-culture methodology with the bacterial solution adhered to the parameters set in Section 2.6.3. Following a 24-hour period, the 48-well plate was removed, rinsed with 0.85% NaCl solution, and subsequently exposed to 2.5% glutaraldehyde, being fixed at 4° for 1 hour in light-protected conditions. Each well was aspirated of the glutaraldehyde solution and sequentially dehydrated with ethanol solutions of increasing concentrations (30%, 50%, 70%, 90%, and 100%) for 10 minutes each. Subsequently, the surface of the specimens in each group underwent gold sputter coating, and bacterial adhesion on the surfaces was observed using Scanning Electron Microscopy (SEM).

2.7 Mechanical property test

2.7.1 Three-point bending test

In adherence to the YY 0270.1-2011 standard, the test span was defined as (50 ± 0.1) mm and the indenter speed was established at 5.0 mm/min. The bending strength, σ , is calculated as follows:

$$\sigma = 3FL/2bh^2$$

where: σ represents the bending strength in MPa; F signifies the maximum force exerted on the specimen, with a unit of N; L indicates the span and is measured in mm; b denotes the width of the specimen in mm; h defines the height of the specimen in mm. Similarly, the bending elastic modulus, E , can be calculated using the formula:

$$E = 3KL^3/4bh$$

where: E represents the bending elastic modulus in MPa; K signifies the maximum slope in the stress-strain curve, expressed in N/mm; L , b , and h retain their previously defined meanings.

2.7.2 Improved three-point bending test

The span was established at (32.0 ± 0.1) mm, and the indenter speed was set to 1.0 mm/min. The formulas used to calculate the maximum stress intensity factor, K_{max} , and the total breaking work, W_b , are as follows:

$$K_{max} = \frac{fP_{max} L_t}{(bth_t^{3/2})} \times \sqrt{10^{-3}} \text{ MPa} \cdot \text{m}^{1/2}$$

$$f(x) = 3x^{1/2} [1.99 - x(1-x)(2.15 - 3.93x + 2.7x^2)] / [2(1+2x)(1-x)^{3/2}]$$

$$x = a/h$$

P_{max} represents the maximum load exerted on the specimen and is measured in N; a delineates the total length of the specimen notch, with units in mm; h_t is the specimen's height, in mm. Additionally, b_t signifies the specimen's width and is also measured in mm, while L_t designates the test span, expressed in mm.

$$W_t = \frac{U}{[2b_t(h_t - a)]} \times 1000 \text{ J/m}^2$$

U represents the area documented under the load/deflection curve, measured in N·mm. The total length of the specimen notch is denoted by a and is expressed in mm. The height and width of the specimen are represented by h_t and b_t respectively, both quantified in mm.

2.7.3 Tensile strength test

Conforming to ISO 527:2012, a 5B sample was utilized, establishing a fixture distance of (20 ± 2) mm and a standard distance of (10 ± 0.2) mm, with a test speed set at 1 mm/min. The tensile strength, β , is defined as $\beta=F/A$, where β is the tensile strength in MPa; F signifies the critical failure force value of the

specimen, measured in N; and A represents the cross-sectional area of the sample, expressed in mm^2 .

2.8 Statistical analysis

Data from each group underwent statistical analysis utilizing GraphPad Prism. Here, 'X' denotes the mean of the data, while S signifies the standard deviation. Subsequent to the normal distribution test and variance homogeneity test, a one-way analysis of variance, coupled with the LSD-t test, was employed for analyzing statistical differences. The P-value threshold was established at 0.05, with $P < 0.05$ indicating statistical significance in the observed differences.

3 Results and discussion

3.1 Characterization of nano-borne Ag-ZrP

Figure 1 shows the synthesis of nano-Ag-ZrP/PMMA composites and the antibacterial mechanism of AgNp. SEM (Loaded voltage is 3kV, and sputtered with Au) reveals that nano-Ag-ZrP exhibits a layered cubic structure (Figure 2A), while Figures 2B–D sequentially depict PMMA materials with the incorporation of 0%wt, 1.5%wt, and 3.0%wt Ag-ZrP, respectively. Notably, in the 1.5%wt group, nano-Ag-ZrP minimally agglomerates within PMMA, showcasing a relatively uniform distribution, whereas the 3.0%wt group experiences an elevation in Ag-ZrP aggregation. It is speculated that the agglomeration of nano-Ag-ZrP in the 3.0%wt group was increased because the

excessive addition of nano-Ag-ZrP led to the increase of the distribution density of nano-Ag-ZrP in PMMA materials, and the enhancement of the electrostatic attraction and van der Waals force, which led to the increase of the agglomeration of nano-Ag-ZrP particles (Sun et al., 2019; Kignelman et al., 2021; Kumar et al., 2022). Figures 2E–H present images of Ag-ZrP, as observed through TEM (Loaded voltage is 200KV, and loaded on copper mesh), at magnifications of 2 μm , 1 μm , 500 nm, and 200 nm, respectively. The ZrP crystal, featuring a sheet structure, is indicated by a blue arrow, and nano silver, exhibiting a black spherical shape, is indicated by a red arrow. Observable from the figure is the loading of nano-Ag particles onto the ZrP surface, bearing substantial morphological resemblance to the nano-Ag-ZrP detailed in existing literature (Qin et al., 2023; Yoshihara et al., 2021; Nocchetti et al., 2022). According to the TEM-Energy Dispersive Spectroscopy (TEM-EDS) element mapping analysis (Figures 2I–N) for P, O, Ag, and Zr elements, an intuitive reflection of each element's distribution within nano-Ag-ZrP is provided, illustrating a uniform dispersion of nano-silver.

Figure 3A displays the FT-IR (400 cm^{-1} ~ 4000 cm^{-1}) spectroscopy analysis of Ag-ZrP, where the peak identified at 3453.78 cm^{-1} corresponds to the symmetric stretching vibration of OH, and the peak at 1637.66 cm^{-1} is attributed to the stretching vibration of OH from water molecules situated between crystal layers (Bhatt et al., 2019). Peaks detected at 1207.58 cm^{-1} and 980.27 cm^{-1} associate with in-plane and out-of-plane vibrations of P-OH groups, respectively (Safari et al., 2016), while those at 1020.94 cm^{-1} and 1105.65 cm^{-1} represent symmetric stretching vibrations of P-O bonds (Yan-Qing et al., 2015). The peak at 746.66 cm^{-1} pertains to P-O-P stretching vibrations (Sinhamahapatra et al., 2010) whereas those at 646.45 cm^{-1} and

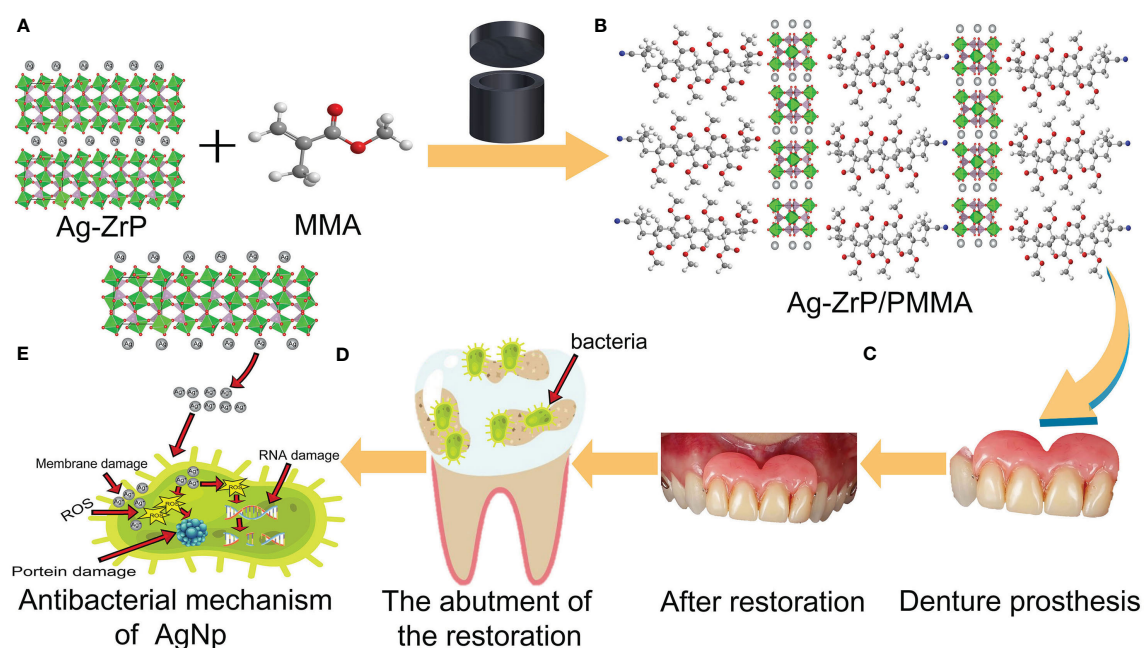


FIGURE 1

(A, B) is the schematic diagram of adding nano-Ag-ZrP to PMMA material; (C) is a denture prosthesis made of nano-Ag-ZrP/PMMA material; (D) for restoration abutments that are not easy to clean and adhere to bacteria; (E) is release Ag^+ for nano-Ag-ZrP and produces antibacterial effect.

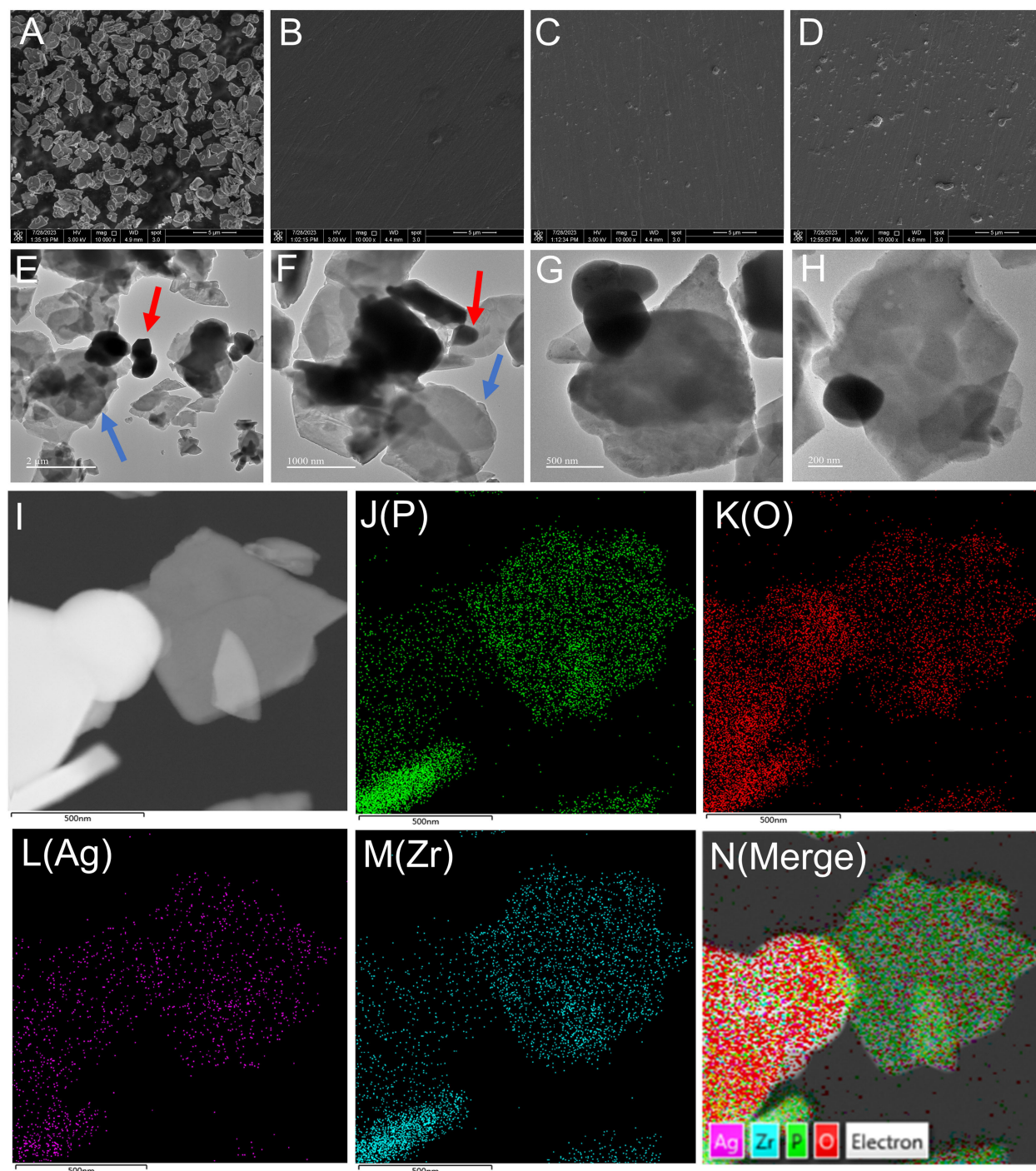


FIGURE 2

(A) is the SEM image of nano-Ag-ZrP; (B–D) are the SEM images of nano-Ag-ZrP/PMMA materials with 0%wt, 1.5%wt and 3.0%wt added in sequence. (E–H) is TEM image of nano-Ag-ZrP; (I–N) is the TEM-EDS element mapping of P, O, Ag and Zr of nano-Ag-ZrP.

548.70 cm^{-1} denote tensile vibrations of Zr-O bonds (Yan-Qing et al., 2015; Xu et al., 2020). Nano-Ag-ZrP was incorporated into PMMA materials in varying mass fractions. The FT-IR spectra for each nano-Ag-ZrP/PMMA composite are presented in Figure 3B. The spectra exhibit peaks at 2995.77 cm^{-1} and 2951.13 cm^{-1} , corresponding to the asymmetric vibration of CH_3 groups (Abutalib and Rajeh, 2020). Additional characteristic peaks include 985.49 cm^{-1} for C-C bond stretching, 1143.98 cm^{-1} for CH_3

bending vibration, 1237.56 cm^{-1} for C-O bond stretching, 1447.97 cm^{-1} for CH_3 deformation, and 1723.04 cm^{-1} for C=O bond stretching (Duan et al., 2008). The peak at 842.16 cm^{-1} is identified as the C=O bond (Abutalib and Rajeh, 2020), with in-plane and out-of-plane flexural vibrations of the C=O bond at 749.24 cm^{-1} (Singhal et al., 2013). Notably, a peak at 550.34 cm^{-1} , attributed to the Zr-O bond stretching, appears in the spectra (Xu et al., 2020). For compositions with nano-Ag-ZrP, a new peak at

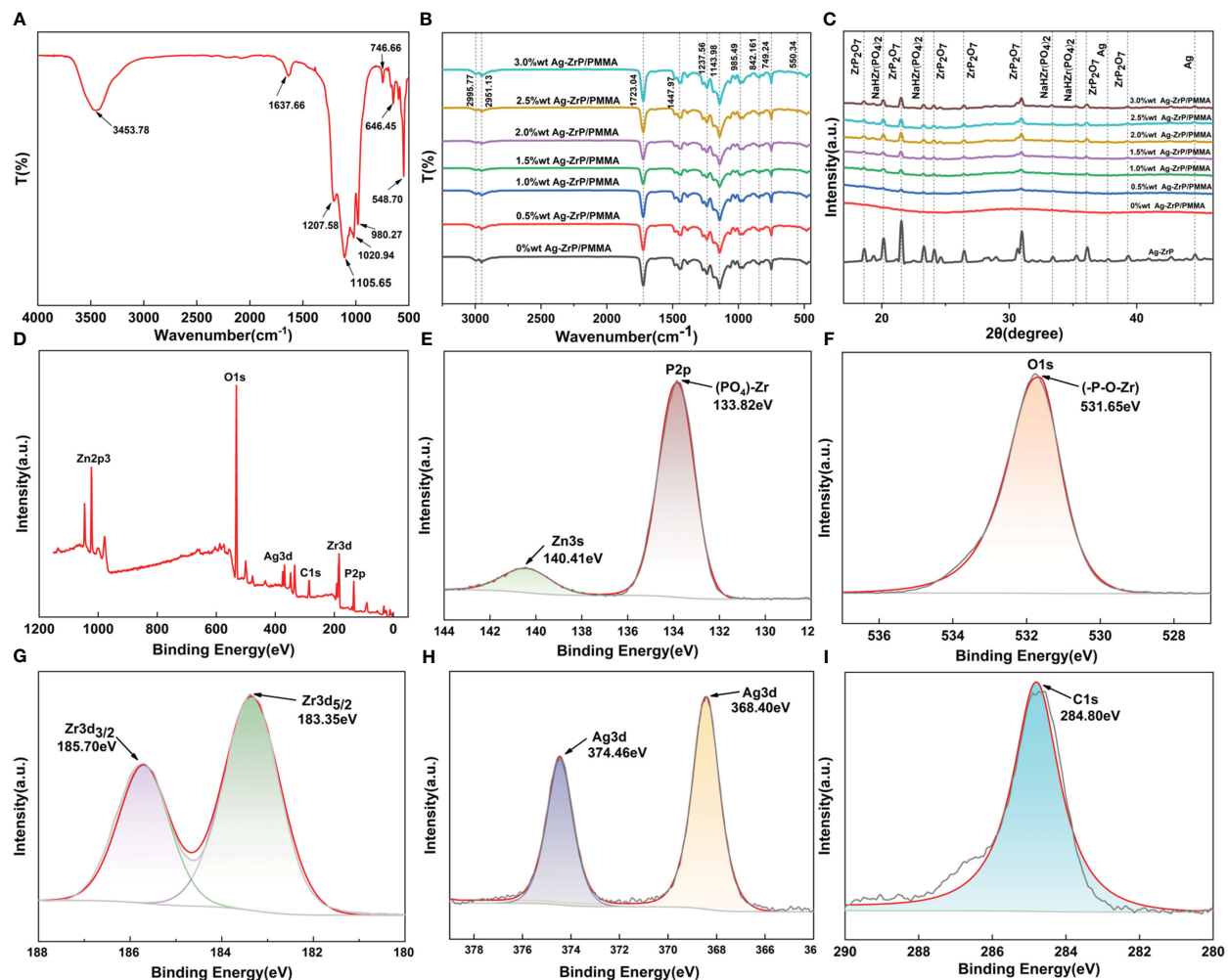


FIGURE 3

(A) is the FT-IR diagram of nano-Ag-ZrP; (B) is the FT-IR diagram of nano-Ag-ZrP/PMMA composites across different groups. (C) is the XRD analysis diagram of nano-Ag-ZrP and different groups of nano-Ag-ZrP/PMMA composites; (D) is the total XPS spectrum of nano-Ag-ZrP; (E-I) are the XPS spectra of P, O, Zr, Ag and C elements.

550.34 cm⁻¹ emerges, becoming increasingly prominent with higher nano-Ag-ZrP concentrations, reaching its maximum at 3.0%wt. These results confirm the successful integration of nano-Ag-ZrP into PMMA materials, with a gradual increase in nano-Ag-ZrP content from 0%wt to 3.0%wt. XRD analysis (The XRD test uses copper targets with a scanning range of 5~70 degrees and a speed of 10 degrees per minute) of nano-Ag-ZrP and each group of nano-Ag-ZrP/PMMA composites (Figure 3C) indicates that peaks at 2θ values of 18.59°, 21.53°, 24.09°, 26.43°, 30.95°, 36.04°, and 39.28° correspond to ZrP₂O₇ (PDF No. 00-010-0004). Characteristic peaks at 20.12°, 23.27°, 33.38°, and 35.24° are aligned with NaHZr(PO₄)₂ (PDF No. 00-055-0206), while those at 37.72° and 44.57° correspond to characteristic peaks of nano silver (PDF No. 04-003-1659). The XRD results for the nano-Ag-ZrP/PMMA composites across different groups reveal a correlation between the concentration of nano-Ag-ZrP and the prominence of characteristic diffraction peaks. Specifically, as the nano-Ag-ZrP content increases, the distinct diffraction peaks corresponding to ZrP₂O₇, NaHZr(PO₄)₂, and Ag become increasingly pronounced.

These peaks are most pronounced in the 3.0%wt group, substantiating the successful integration of nano-Ag-ZrP into the PMMA materials. XPS (The results of XPS were analyzed by Avantage software) global spectrum analysis of nano-Ag-ZrP is presented in Figure 3D. The binding energies at 1022.80 eV and 1044.52 eV correspond to Zn2p_{1/2} and Zn2p_{3/2} orbits, respectively (Mahakal et al., 2023). According to the reagent manufacturer, the Ag-ZrP antibacterial agent incorporates a minimal quantity of zinc oxide (approximately 5.0~10.0%wt), primarily functioning as a dispersant, and the peak at 248.80 eV signifies the charge correction peak of C1s. Figures 3E-I sequentially exhibit the XPS spectra of P, O, Zr, Ag and C elements. The figure highlights that the peak of P2p at 133.82 eV corresponds to P⁵⁺, representing the (PO₃)-Zr bond (Yan-Qing et al., 2015; Yuan et al., 2005), while the peak at 140.41 eV emerges as the interference peak of Zn3s. The peak of 531.65 eV aligns with O1s, corroborating the binding energy of the (-P-O-Zr) bond (Pourbeyram, 2016). Figure 3G elucidates the XPS map of Zr, where Zr3d demonstrates two distinctive peaks: 183.35 eV representing Zr3d_{5/2} and 185.70 eV indicative of Zr3d_{3/2},

denoting Zr^{4+} and indicating covalent connections between Zr^{4+} and PO_4^{3-} as substantiated by related research (Yan-Qing et al., 2015; Chen et al., 2022; Li et al., 2021). Additionally, AgNP exhibits two prominent peaks, with 368.40 eV pertaining to $Ag3d_{5/2}$ and 374.46 eV to $Ag3d_{3/2}$ (Xu et al., 2020; Biswal et al., 2011). The comprehensive analysis through SEM, TEM, FT-IR, XRD, and XPS preliminarily substantiates that the material can be classified as nano-Ag-ZrP, and Ag-ZrP has been successfully integrated into the PMMA materials.

3.2 Characterization and identification of primary HGFs

On day 8, primary HGFs were observed to migrate from the gingival tissue (Figure 4A), and by day 15, the cells exhibited a swirl-like arrangement (Figure 4B). Figure 4C illustrates the morphology of fifth-generation HGFs under the microscope (40x magnification), revealing spindle- or star-shaped cells with centrally located nuclei, clear nucleoli, and round or oval nuclear shapes, consistent with typical fibroblast characteristics. Immunostaining was performed on the fifth-generation HGFs utilizing anti-vimentin antibody and anti-cytokeratin antibody (Cytokeratin 8). Figures 4D-I display immunofluorescence staining, while Figures 4J, K depict immunohistochemical staining. Staining for Vimentin was positive, whereas Cytokeratin 8 staining was negative. These results align with the staining findings for HGFs by Xuqian Liu, Kai Yang et al., confirming the cells to be mesodermal-origin fibroblasts (Liu et al., 2016; Yang et al., 2021).

3.3 Effect of nano-Ag-ZrP on biosafety of room-temperature cured PMMA materials

AgNP has been documented to exert specific impacts on cell activity, oxidative stress, and cytoskeletal protein among other factors (Shimizu et al., 2022; Perde-Schrepler et al., 2019) necessitating further investigation into the biosafety of Ag-ZrP/PMMA materials. Figure 5A illustrates the outcomes of apoptosis detection via Annexin V-FITC/PI double staining, following a 3-day culture of HGFs with the extract from each sample group. Subsequent statistical analysis of the apoptosis findings (Figure 5C) revealed a notable increase in the early apoptosis rate of HGF-s in the 3.0%wt group compared to the 0%wt group ($P < 0.05$). In comparison to the 0.5%wt group, the late apoptosis/necrosis rates of HGFs in the 2.5%wt and 3.0%wt groups notably escalated ($P < 0.05$), yet remained statistically indistinguishable among other groups. A plausible reason for the augmented apoptosis rate in 2.5%wt and 3.0%wt groups could be attributed to a minor release of AgNP with the elevation of Ag-ZrP supplemental level, thereby inducing a modest cytotoxicity. Despite this, the majority of cells maintained normal activity, and the overarching apoptosis rate did not reach substantial levels, warranting the incorporation of additional detection methods for accurate determination. Figure 5B portrays the staining results of live and dead cells post a 3-day culture of HGFs in each group's extract solution,

demonstrating that an uptick in nano-Ag-ZrP supplemental level correlated with an elevation in the count of live cells across all experimental groups and a reduction in the proportion of dead cells, without overt cell morphological alterations across groups. The CCK-8 method, employed to ascertain the activity of HGFs post a 3-day culture in extracts from each group (Figure 5D), yielded no significant discrepancy in the Relative Growth Rate (RGR) of HGFs among extract groups ($P > 0.05$), with an RGR of HGFs exceeding 85% following 24h, 48h, and 72h of culture. According to the cytotoxicity grading evaluation criteria stipulated by ISO 10993-5:2009, the cytotoxicity of extracts from each group post 24h, 48h, and 72h of cultured HGFs consecutively could be classified as less than or equal to grade 1, with no discernible cytotoxicity detected. Erkoş and Jie SUN et al. incorporated AgNP into PMMA denture base materials, while Cecilia et al. introduced AgNP-GO into PMMA denture base materials, with neither finding substantial cytotoxicity in PMMA materials containing a low mass fraction of AgNP (Bacali et al., 2020; Kurt et al., 2017; Sun et al., 2021). Consequently, it may be inferred that the biosafety of room-temperature cured PMMA materials remains favorable when the addition of nano-Ag-ZrP is restrained below 3.0%wt.

3.4 Effect of nano-Ag-ZrP on antibacterial properties of room-temperature cured PMMA

Table 1 illustrates the colony recovery number and antibacterial rate of *S. mutans* and *E. coli* across varied sample groups. The colony-counting results for *S. mutans* (Figure 6A) and *E. coli* (Figure 6B) elucidate that an elevation in nano-Ag-ZrP content correspondingly reduces the colony number of sample elutes across all groups. A diminution to the least colonies of *S. mutans* and *E. coli* is observed at 3.0%wt. Figure 6E reveals the statistical analysis results of *S. mutans* recovered colony numbers across different groups. A substantial reduction in the *S. mutans* colony number is observed in all groups compared to the control group ($P < 0.05$). Pairwise comparison among experimental groups reveals statistically significant discrepancies ($P < 0.05$) among them, save for the comparisons between 2.0%wt and 2.5%wt, 2.0%wt and 3.0%wt, and 2.5%wt and 3.0%wt ($P > 0.05$). For *E. coli*, statistical results of recovered colony numbers (Figure 6F) demonstrate a notable reduction in colony numbers across all experimental groups compared to the 0%wt control group ($P < 0.05$), with all experimental group pairwise comparisons showcasing statistically significant disparities ($P < 0.05$). Research by Shenggui Chen et al. involved the addition of 0.1–0.25%wt AgNP-CNCs (nanocellulose) to PMMA materials (Chen et al., 2018), Souza Neto et al. introduced 0.05%wt AgNP to PMMA resin material (Souza Neto et al., 2019), and Sultan Aati et al. incorporated silver-carrying mesoporous SO_2 nanoparticles into the denture base (Aati et al., 2022). All studies affirm that materials embedded with AgNP can substantially enhance the antibacterial capability of PMMA materials, aligning with the findings obtained in this study that an increased AgNP content improves antibacterial effects. SEM images depicting *S. mutans* adherence to the surface of specimens in each group are

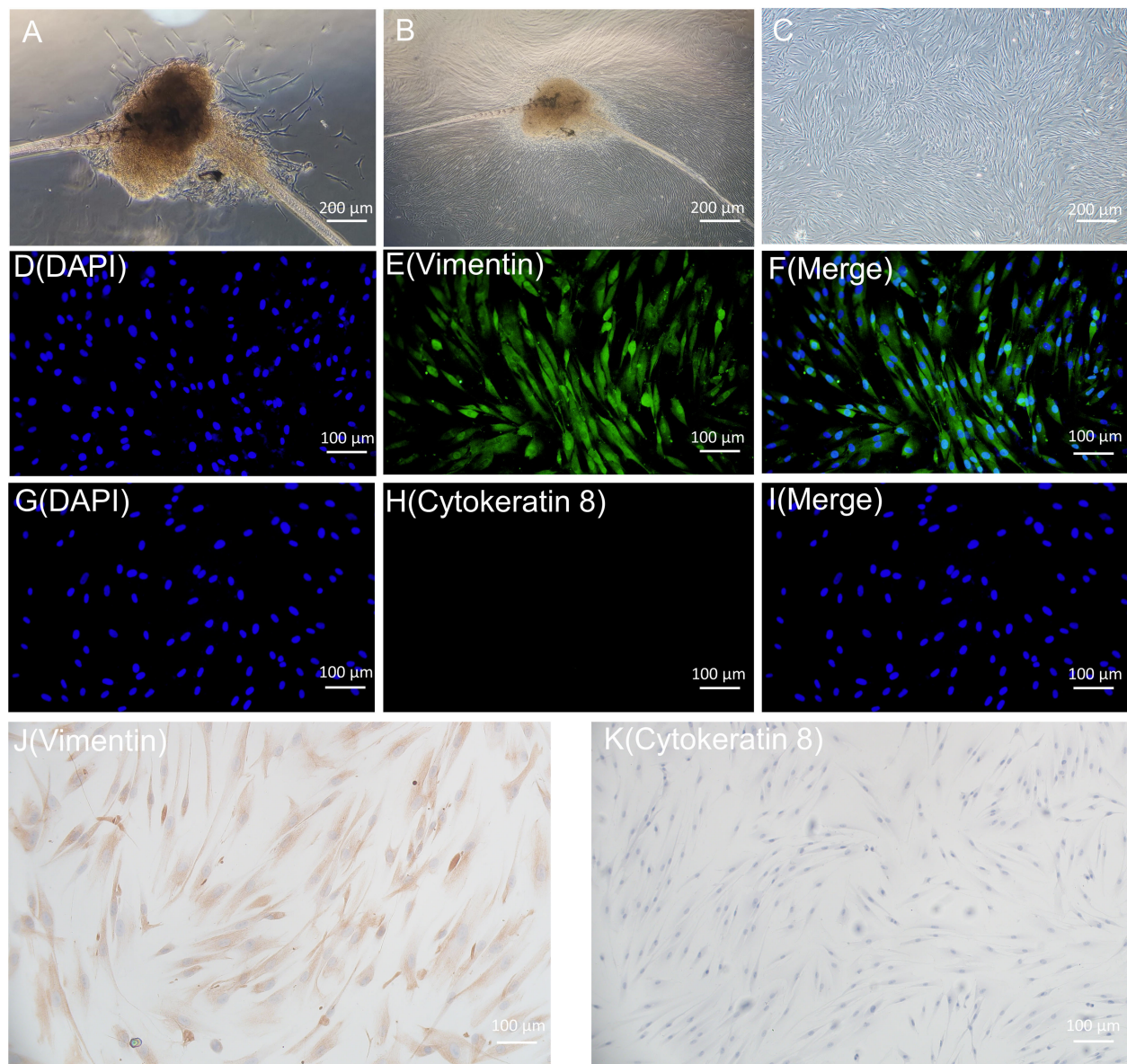


FIGURE 4
(A, B) are the primary HGFs flowing out of tissue blocks. C is the microscopic morphology of 5th generation HGFs. (D–I) is the result of immunofluorescence staining of HGFs; (J, K) were immunohistochemical staining results of HGFs.

presented in Figure 6C. *S. mutans* in the 0%wt~0.5%wt group exhibits a smooth surface and a continuous, intact envelope (indicated by white arrows), contrasted by the 1.0%wt group where the envelope appears wrinkled and sunken. Within the 1.5% ~ 3.0%wt groups, the membrane of *S. mutans* evidences varied degrees of perforation and damage (indicated by white arrows). Figure 6D discloses SEM observation images of *E. coli* on specimen surfaces across groups. As you can see from the arrows, *E. coli* in the 0%wt~0.5%wt group manifests well-preserved morphology, with a predominantly smooth surface and negligible folding and collapse. However, in the 1.0%wt and 1.5%wt groups, the *E. coli* cell membrane surface becomes notably rough and wrinkled. A further increase in the nano-Ag-ZrP antibacterial agent leads to an escalation in *E. coli* surface folds, cell membrane

rupture, and exposure of bacterial contents in the 2.0% to 3.0%wt group. The SEM images of *S. mutans* and *E. coli* on the surface adhesion of specimens of each group showed that the damage degree of *S. mutans* and *E. coli* on the surface adhesion of specimens of each group was gradually serious with the increase of nano-Ag-ZrP addition. It is shown that the antibacterial properties of PMMA materials can be improved by adding nano-Ag-ZrP to PMMA materials. The presumed antibacterial mechanism may be attributed to direct contact between AgNP and the bacterial cell membrane, Ag⁺ release, bacterial cell wall and cell membrane disruption, reactive oxygen species (ROS) generation, bacterial substance leakage, and subsequent bacterial internal structure obliteration, culminating in a bactericidal effect (Ji et al., 2020; Hajipour et al., 2012; Dakal et al., 2016; Estevez et al., 2021).

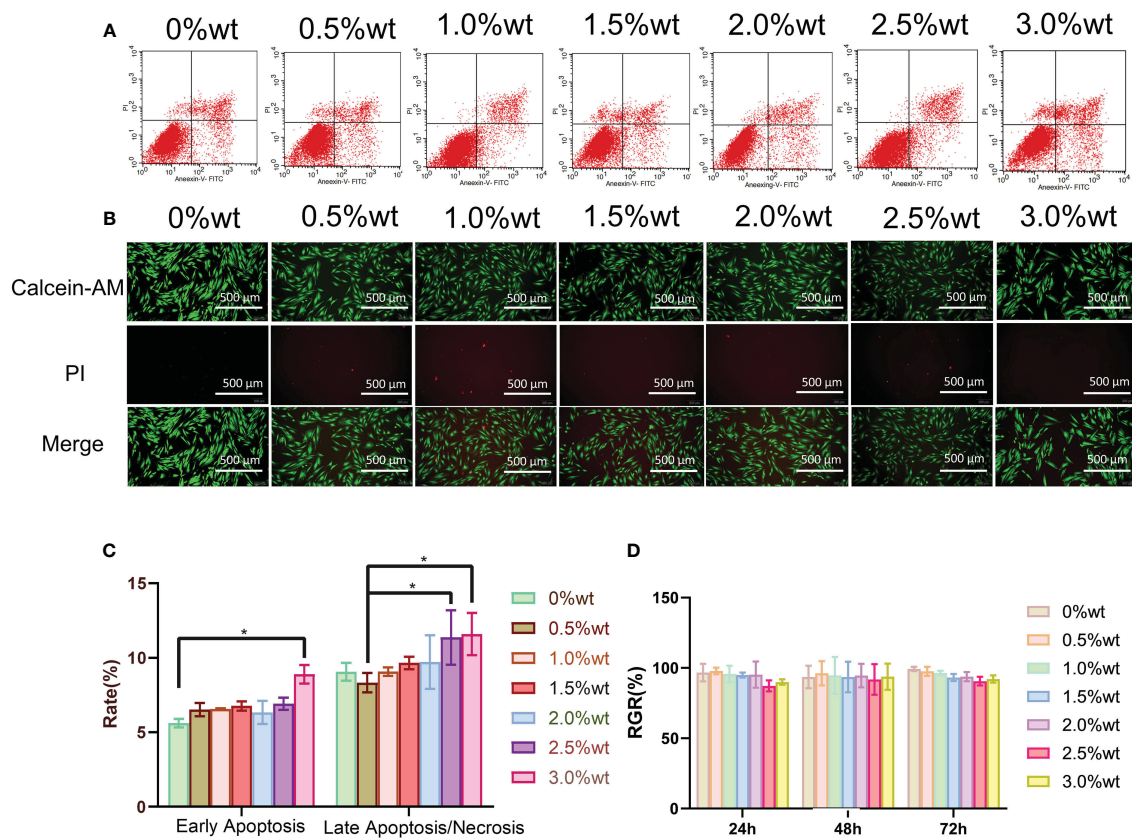


FIGURE 5

(A) is the Annexin V-FITC/PI double staining was used to detect apoptosis. (B) is the staining results of live and dead cells after incubation with HGFs 3 days in the extract solution of each group. (C) is the analysis of apoptosis detection results; (D) is the detection result of CCK-8 method. * symbol 0.01<P-value<0.05, it is indicating significant differences.

SYTO 9 has the capability to stain the nucleic acid of live bacteria, or bacteria possessing damaged cell membranes, resulting in green fluorescence emission, while propidium iodide (PI) can stain the nucleic acid of dead bacteria, or bacteria with compromised cell membranes, to emit red fluorescence. Upon concurrent application, bacteria with impaired cell membranes exhibit yellow fluorescence. Figure 7 illustrates the staining results for live and dead bacteria of *S. mutans* and *E. coli*. It is evident from the figure that, concomitant with the augmentation of nano-

Ag-ZrP antibacterial agent content, the quantity of live *S. mutans* and *E. coli* bacteria on the specimen surface progressively diminished, whereas the number of dead bacteria or bacteria with damaged cell membranes progressively escalated. When the addition of Ag-ZrP antibacterial agent reached 2.5%wt, and subsequently 3.0%wt, the dead bacteria or bacteria with compromised cell membranes pervaded almost the entire visual field on the specimen surface, aligning with the outcomes from the film coating experiment.

TABLE 1 The number of *S. mutans* and *E. coli* colonies recovered and the antibacterial rate of each group.

Additive amount (wt, %)	Colony count ($\times 10^4$ CFU/ml, $\bar{X} \pm S$)		Antibacterial rate (R, %)	
	<i>S. mutans</i>	<i>E. coli</i>	<i>S. mutans</i>	<i>E. coli</i>
0	216.70 $\pm$ 10.50	610.30 $\pm$ 16.26	–	–
0.5	100.70 $\pm$ 6.50	417.30 $\pm$ 16.50	53.53	31.62
1.0	71.33 $\pm$ 2.51	304.30 $\pm$ 12.58	67.08	50.14
1.5	36.33 $\pm$ 2.51	219.70 $\pm$ 8.15	83.23	64.00
2.0	14.33 $\pm$ 2.08	152.00 $\pm$ 9.00	93.38	75.09
2.5	9.00 $\pm$ 2.00	83.67 $\pm$ 6.11	95.85	86.30
3.0	4.33 $\pm$ 1.52	42.67 $\pm$ 4.04	98.00	92.98

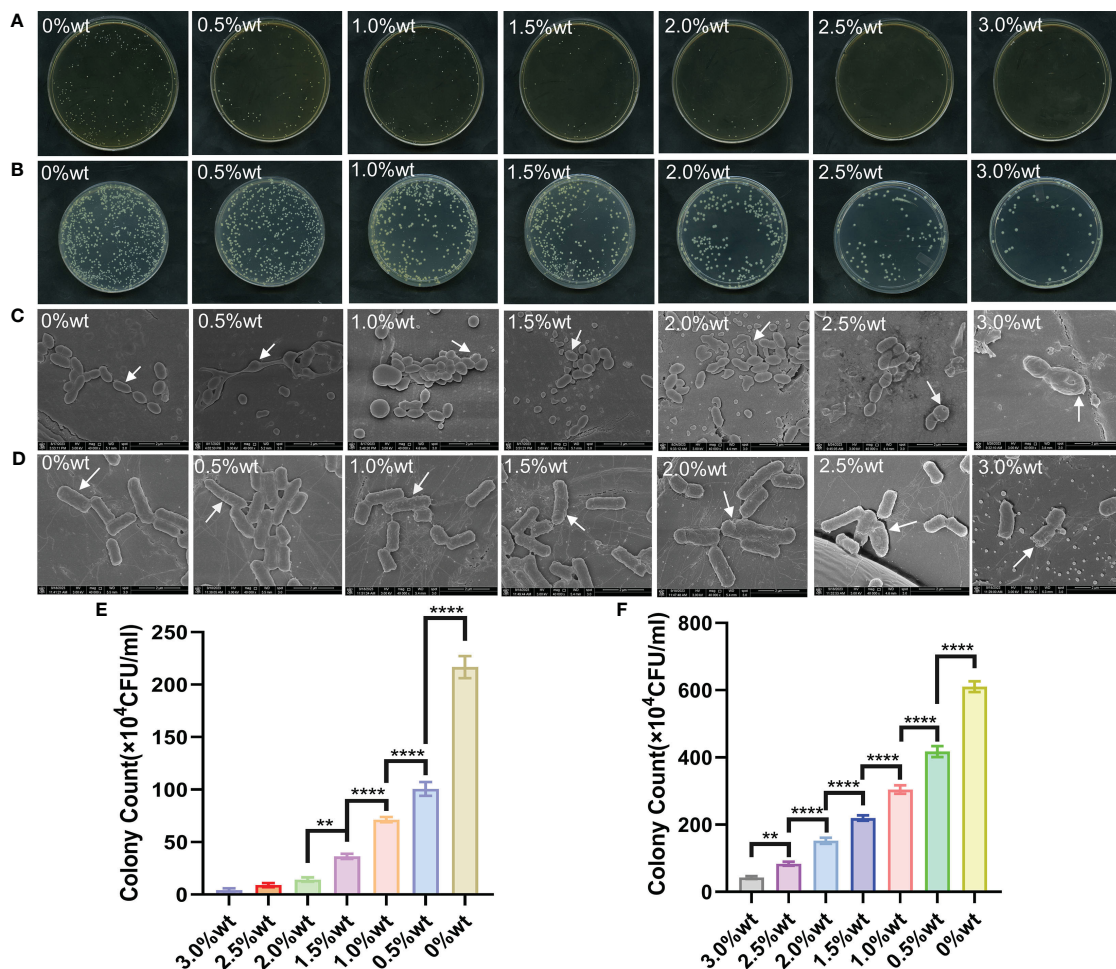


FIGURE 6

(A) is the plate colony count result of *S. mutans*. (B) is the colony count result of *E. coli*. (C) is the SEM image of *S. mutans* on the surface of each group of specimens. (D) is the SEM image of *E. coli* on the surface of each group of specimens; (E) is the results of statistical analysis of the colony count of *S. mutans*. (F) is the result of statistical analysis of *E. coli* colony count. * symbol $0.01 < P\text{-value} < 0.05$, it is indicating significant differences; ** symbol $0.001 < P\text{-value} < 0.01$, it is indicating that the difference is very significant; **** symbol $P\text{-value} < 0.0001$, it is indicating that the significance of the difference is the highest.

3.5 Effect of nano-Ag-ZrP on mechanical properties of PMMA materials

The fracture morphology of the sample, post-three-point bending test fracture, as visualized in the SEM image (Figure 8A),

reveals that the cross-section of the sample in the 0%wt group is predominantly smooth with a flat, continuous fracture line, indicative of typical brittle fracture characteristics. With the escalation of nano-Ag-ZrP content, a gradual increase in the number of nano-Ag-ZrP crystals on the fracture surface of the 0.5%

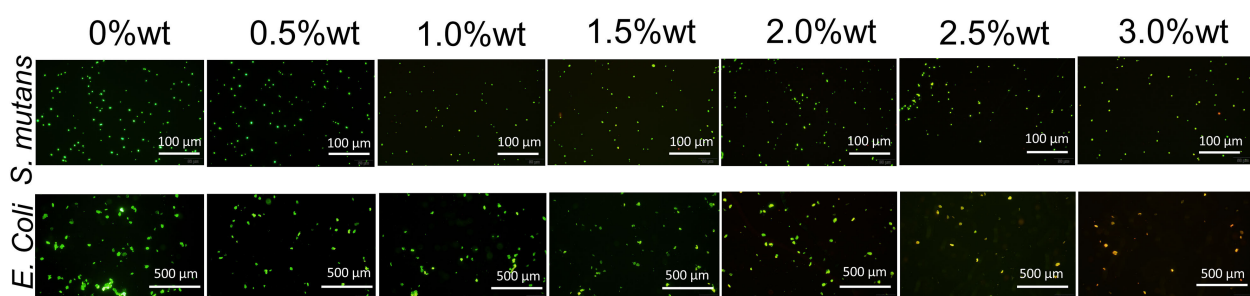


FIGURE 7

The staining results of *S. mutans* and *E. coli* on the surface of each group of specimens.

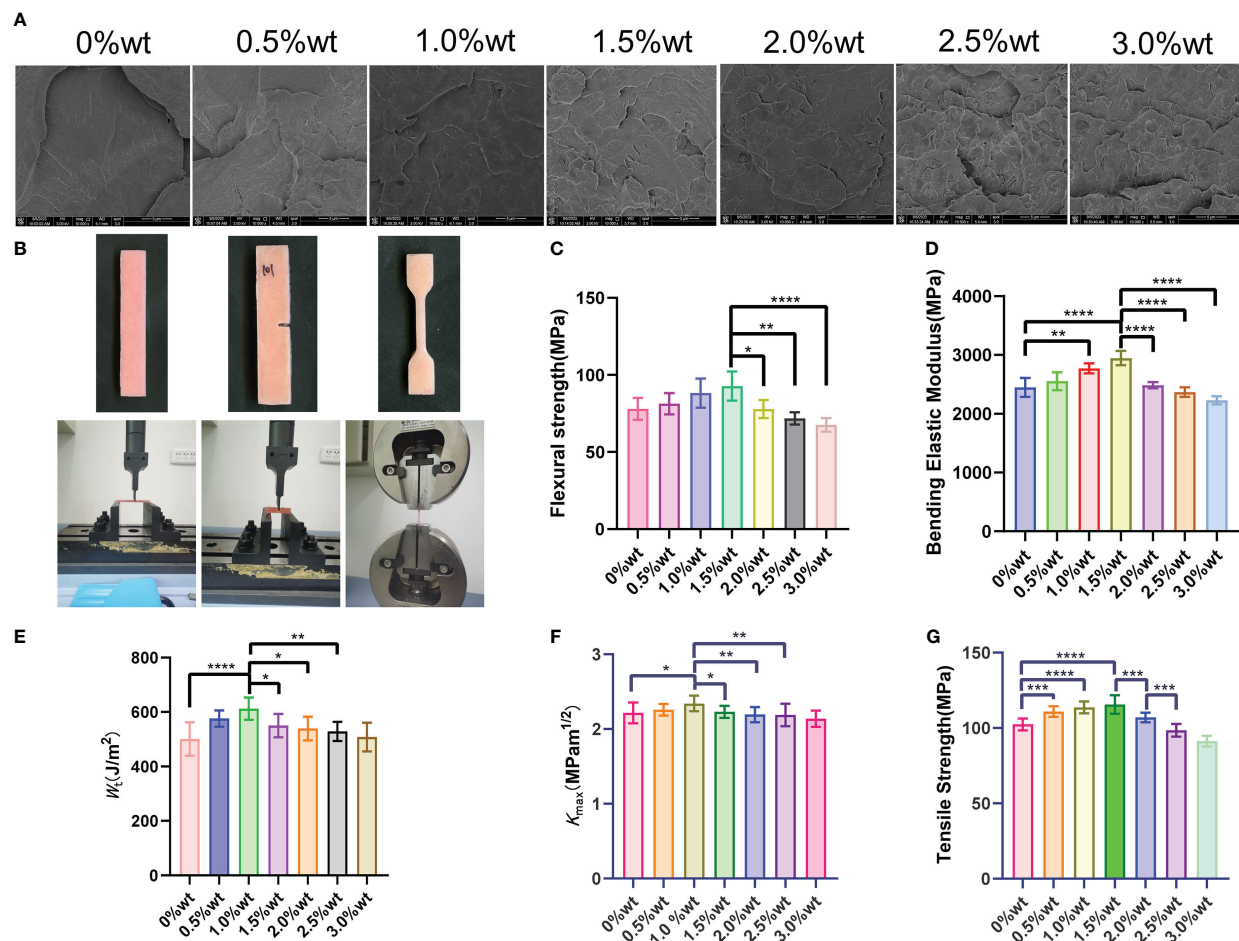


FIGURE 8

(A) is SEM observation of three-point bending specimen; (B) is the specimen shape and universal mechanical testing machine; (C, D) are the results of three-point bending test; (E, F) are the results of improved three-point bending test; (G) is the result of tensile test. * symbol $0.01 < P\text{-value} < 0.05$, it is indicating significant differences; ** symbol $0.001 < P\text{-value} < 0.01$, it is indicating that the difference is very significant; *** symbol $0.0001 < P\text{-value} < 0.001$, it is indicating difference is more significant; **** symbol $P\text{-value} < 0.0001$, it is indicating that the significance of the difference is the highest.

wt ~ 3.0%wt group is observable, and the evident rough stratification of the fracture surface displays dimple formation. Nano-Ag-ZrP particles were identified within the dimple, exhibiting classic ductile fracture traits. A notably deep and large dimple is present on the fracture surface of the 0.5%wt ~ 2.00%wt group. Conversely, the dimple on the fracture surface of the 2.5wt ~ 3.0%wt group is shallow and small, with Ag-ZrP crystal particles appearing more agglomerated. Figure 8B illustrates the shape of the mechanical properties test specimen and the universal mechanical testing machine. Three-point bending test results (Figures 8C, D) 1.5%wt, the bending strength and bending elastic modulus of the PMMA material incrementally rise, subsequently diminishing with a continuous increase of Ag-ZrP content. Both the bending strength and bending elastic modulus of the 1.5%wt group reached peak values. When compared with the 1.5%wt group, the 2.0%wt, 2.5% wt, and 3.0%wt groups exhibit significantly reduced bending strength and bending elastic modulus, with the difference being statistically significant ($P < 0.05$). Figures 8E, F depict the outcomes from the modified three-point bending test, illustrating a notable

trend in the W_t and K_{max} of each group with varying nano-Ag-ZrP content. Specifically, in the range of 0%wt to 1.0%wt nano-Ag-ZrP, both W_t and K_{max} exhibit a gradual increment, showcasing a statistically significant difference in comparison to the 0%wt control group ($P < 0.05$). Conversely, in the 1.0%wt to 3.0%wt nano-Ag-ZrP range, a gradual decline in the W_t and K_{max} of the sample is observed, with the zenith being at the 1.0%wt group. The tensile test results from each group's specimens (Figure 8G) demonstrate a gradual ascending trend in the tensile strength (TS) within the 0%wt to 1.5%wt group, peaking at 1.5%wt. Subsequently, a gradual descending trend in TS is exhibited by the experimental groups, nadiring at 3.0%wt. Rongrong CHEN et al. integrated 3.0wt% nano-Ag-ZrP (Novaron) into a blend comprising PMMA powder, MMA monomer, silanized nano-zirconia, and silanized aluminum borate whisker, determining that an enhancement in the bending strength and hardness of PMMA material was realized exclusively post nano-Ag-ZrP addition (R. Chen et al., 2017). Wenbo Liao et al., found that the addition of silanized nano-Ag-ZrP to PMMA material resulted in an initial

increase followed by a decrease in the bending strength as the mass fraction of Ag-ZrP augmented from 0wt% to 3.0wt%. A pinnacle in the bending strength of PMMA material was achieved when the nano-silver zirconium phosphate was introduced at 1.0%wt (Liao et al., 2020). Incorporation of nanoparticles, such as zirconium dioxide (ZrO₂), graphene oxide (GO), and silicon dioxide (SiO₂), into PMMA materials has been investigated by various scholars, revealing a general tendency: an initial enhancement in mechanical properties bending strength, bending elastic modulus, and tensile strength of PMMA materials, followed by a decrement (Chęcinska et al., 2022; Sahm et al., 2023; Ahamad Said et al., 2022; Alzayyat et al., 2022; Wang et al., 2019). Literature elucidates that the mechanism underpinning the nanoparticle-induced enhancement of mechanical strength in PMMA materials involves the induction of stress aggregation by the nanoparticle crystal particles. Upon integration into PMMA materials, these particles promote silver patterns in the surrounding matrix, instigating plastic deformation of the adjacent matrix, thereby absorbing external energy and generating toughness. Concurrently, nanoparticles are also capable of terminating the silver lines, yielding, and imparting a toughening effect. However, an incessant increase in the nano-Ag-ZrP crystal content compromises the dispersion of nanoparticle crystals in PMMA material, initiating agglomeration. Transformed from small to large particulate matter through agglomeration, the nanoparticle crystals reduce in surface area, forming a stress concentration zone, and their adhesion to PMMA material deteriorates. The manifestation of numerous defective structures within the material consequently undermines the mechanical properties of PMMA materials (Daelemans et al., 2016; Zeidi et al., 2023; Wang et al., 2017; He et al., 2018). In the current experiment, SEM observation of the fracture surface of each group of specimens, coupled with analysis post-mechanical properties test, affords a viewpoint aligning with preceding studies.

4 Conclusion

The morphological characteristics, functional groups, crystal structures, and elemental composition of commercially available nano-Ag-ZrP were analyzed employing SEM, TEM, FT-IR, XRD, and XPS. The material, initially presumed to be nano-Ag-ZrP, demonstrated optimal antibacterial activity against *S. mutans* and *E. coli* when incorporated at a 3.0%wt into the room-temperature cured PMMA material, achieving antibacterial rates of 98.00% and 92.98%, respectively. A concentration of 2.0%wt resulted in antibacterial rates of 93.38% against *S. mutans* and 75.09% against *E. coli* in the PMMA material. Mechanical property testing revealed that room-temperature-curing PMMA materials exhibited peak mechanical properties when the concentration of nano-Ag-ZrP antibacterial agent was within 1.0%~1.5%wt. When the antimicrobial agent concentration was increased to 2.0%wt, the mechanical strength of the PMMA material did not diminish compared to the 0%wt group, while concentrations starting from 2.5%wt resulted in reduced mechanical strength relative to the 0% wt group. Furthermore, cytotoxicity was not detected at nano-Ag-ZrP concentrations below 3.0%wt, indicating commendable

biosafety. Consequently, future research aiming to enhance the antimicrobial and mechanical properties of PMMA materials should prioritize augmenting antimicrobial properties without compromising the mechanical integrity of the PMMA materials. In summary, it is recommended that the incorporation of nano-Ag-ZrP (RHA-1F-II) in room-temperature curing PMMA material be maintained within 1.5%wt ~ 2.0%wt.

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 Stomatology Hospital of Jilin University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

XC: Writing – original draft, Writing – review & editing. TY: Data curation, Methodology, Supervision, Writing – review & editing. SS: Methodology, Writing – review & editing. AL: Conceptualization, Data curation, Writing – review & editing. XW: Formal Analysis, Funding acquisition, Methodology, 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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Lipid nanoparticles based on natural matrices with activity against multidrug resistant bacterial species

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Lately, the bacterial multidrug resistance has been a reason to public health concerning around world. The development of new pharmacology therapies against infections caused by multidrug-resistant bacteria is urgent. In this work, we developed 10 NLC formulations composed of essential oils (EO), vegetable butter and surfactant. The formulations were evaluated for long-term and thermal cycling stability studies in terms of (particle size, polydispersion index and Zeta potential). *In vitro* antimicrobial assays were performed using disk diffusion test and by the determination of the minimum inhibitory concentration (MIC) performed with fresh and a year-old NLC. The most promising system and its excipients were structurally characterized through experimental methodologies (FTIR-ATR, DSC and FE-SEM). Finally, this same formulation was studied through nanotoxicity assays on the chicken embryo model, analyzing different parameters, as viability and weight changes of embryos and annexes. All the developed formulations presented long-term physicochemical and thermal stability. The formulation based on cinnamon EO presented *in vitro* activity against strains of *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolated from humans and *in vivo* biocompatibility. Considering these promising results, such system is able to be further tested on *in vivo* efficacy assays.

KEYWORDS

multi-resistant bacteria, nanostructured lipid carriers, vegetable butter, essential oil, natural antibiotics

1 Introduction

The emergence of multi-resistant bacteria (MDR) to antibiotics represents one of the principal global threats to public health (Murray et al., 2022). The clinicians have been considered the use of the last therapeutics options, such as vancomycin, polymyxin B, carbapenems and colistin, with low rates of success (Wang et al., 2022; Yao et al., 2023). The infections by MDR are currently associated with considerable increase in morbidity and mortality of intensive care units (Braga et al., 2018), increasing hospital costs (Osme et al., 2020) and resistance against the strategies used to combating them, as antibiotics association (Batchelder et al., 2023). There is an estimative that by 2050 will there will be 10 million deaths from MDR, annually (O'Neill, 2016).

In addition, in the COVID-19 pandemic, there was an indiscriminate use of broad-spectrum antibiotics (Freire et al., 2023), increasing the antimicrobial resistance in worldwide (Kariyawasam et al., 2022), as reported for bacterial resistance to carbapenems (Pan American Health Organization, 2021).

In this sense, the use of compounds of natural origin, such as essential oils (EO) can represent an interesting alternative to combat infections by MDR pathogens. EO are promising drug candidates, once they have several intrinsic biological properties, such as antibacterial, antifungal, antiviral, antioxidant, anticancer, analgesic, anti-inflammatory, biopesticide, repellent, among others (Elshafie and Camele, 2017; Cimino et al., 2021). These compounds come from the secondary metabolism of plants, being extracted from bark, leaves, flowers, buds, seeds, roots and fruits. Its chemical constitution is complex, being mainly composed of monoterpenes, sesquiterpenes and terpenoids (El Asbahani et al., 2015). However, the application of EO in the clinical approaches is limited, due to its low solubility in water, low physicochemical stability, high volatility and hydrolysis (Carbone et al., 2018; Guidotti-Takeuchi et al., 2022). Moreover, the toxicity of the esophageal and gastrointestinal mucosa limits its use by enteral administration route, as well as allergic reactions can be observed in the skin if they are insufficiently diluted (Lammari et al., 2021).

Thus, the EO nanoencapsulation can be an interesting alternative to overcome such limitations. The lipid nanoparticles, including the nanostructured lipid carriers (NLC), which is composed by solid and liquid lipids at room temperature (Ribeiro et al., 2021), seems to be an excellent strategy. NLC are biodegradable, biocompatible, scalable and ecofriendly (Dupuis et al., 2022). Such systems allow the encapsulation of hydrophobic molecules as EO, improving its physicochemical stability (Cimino et al., 2021), protecting against degradation and evaporation. This process still increases the EO solubility in water, optimizing its bioavailability, allowing its sustained release (Katopodi and Detsi, 2021) and, consequently, decrease the EO intrinsic toxicity (Dajic Stevanovic et al., 2020).

This work reports the development of NLC formulations based on natural matrices composed of vegetable butter and EO with activity against strains of *Acinetobacter baumannii*, *Klebsiella*

pneumoniae and *Pseudomonas aeruginosa* isolated from humans. These bacterial species were selected because they are drug multi-resistant, including the resistance to antibiotics considered the last therapeutic choice. Consequently, they are the most prevalent species of the most of infections described in clinics, increasing hospital costs and mortality rates (Tacconelli et al., 2018; Osme et al., 2020; World Health Organization, 2021; Murray et al., 2022; Wang et al., 2022; Micheli et al., 2023; Yao et al., 2023). Firstly, a screening of 14 natural oils was carried out to select those with the most promising *in vitro* antibacterial activity as the structural and bioactive excipient in the preparation of different NLC formulations. The resultant formulations were monitored by a long-term physicochemical stability study (1 year, 25°C), followed by thermal cycling stability test. Then, the *in vitro* antibacterial activity was evaluated through the determination of the minimum inhibitory concentration (MIC). Finally, the structural characterization and the *in vivo* nanotoxicity test on chicken embryo model (CE) were performed to determinate compatibility between the excipients, thermal stability and the safety of the most promising nanosystems.

2 Materials and methods

2.1 Materials

Ucuuba butter, cinnamon bark EO (*Cinnamomum zeylanicum*), oregano EO (*Origanum vulgare*), tea tree EO (*Melaleuca artemisiaefolia*), peppermint EO (*Mentha piperita*), rosemary EO (*Rosmarinus officinalis*), clove EO (*Eugenia caryophyllus*), ginger EO (*Zingiber officinale*), salvia EO (*Salvia sclarea*), basil EO (*Ocimum basilicum*), citronella EO (*Cymbopogon nardus*), star anise EO (*Illicium verum*), frankincense EO (*Boswellia carterii*), candeia (*Eremanthus erythropappus*) and copaiba (*Copaifera officinalis*) vegetable oils were commercially obtained from the company Engetec/ Engenharia das Essências®, Brazil. Poloxamer (Kolliphor® P188), used as a surfactant, was purchased from Sigma-Aldrich, USA. Deionized water (18 MΩ) was obtained from a Milli-Q® Direct 8 ultra-pure water purifier. Bacterial isolates of *A. baumannii* (65JF, OXA-23 and POA4-28), *K. pneumoniae* (ATCC700603, Responsive and BacTray) and *P. aeruginosa* (IMP-1 and PAO-1) were obtained from the collection of the Molecular Microbiology Laboratory of the Federal University of Uberlandia, Brazil. *Klebsiella pneumoniae* ATCC700603 is a standard strain. Responsive *K. pneumoniae* was isolated from a hospitalized patient in Uberlandia – MG that we used as a secondary control for sensitivity to antibiotics. All the other strains were isolated from hospitalized patients in Brazil and characterized as MDR, once they came from an acquired resistant infection not susceptible to at least 1 agent in 3 or more antimicrobial categories (Magiorakos et al., 2012). The medium culture used were: Soy Tryptone Agar (Isofar, Brazil), MacConkey Agar (Ion, India), Mueller Hinton Agar (Laboratorios Conda S.A., Spain), Mueller Hinton Broth (Biolog®). Disks impregnated with Polymyxin B were purchased from Cefar Diagnóstica Ltda, São Paulo, Brazil.

2.2 Screening of vegetable and essential oils

The screening of different vegetable and EO was carried out through the disk diffusion test against *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* strains. Commercial polymyxin B disks were used as a positive control. The strains of interest were inoculated in Soy Tryptone agar (TSA) or MacConkey agar medium culture, separately, and incubated at 37°C for 24 h. After this period, isolated colonies of 3 to 5 species of the same morphological type were collected and diluted in 10 mL of 0.9% sterile saline solution, with turbidity adjusted according 0.5 MacFarland scale, up to a final concentration of colony forming units 1.5×10^8 (CFU) mL⁻¹ (Ribeiro et al., 2021). The resulting bacterial suspension was used in the disk diffusion test (pure EO, screening stage) and in the MIC determination assay. Plates containing Mueller Hinton agar were previously prepared. With a sterile swab, each bacterial suspension analyzed (*A. baumannii*, *P. aeruginosa* and *K. pneumoniae*) was equally distributed over the agar surface and stored by 5 min (25°C). After this period, disks impregnated with polymyxin B (positive control) and EO were positioned over the agar, in duplicate. Approximately 20 µL of each pure oil was added to sterile filter disks (6 mm in diameter) immediately before being positioned on the plates. The plates were incubated at 37 ± 1 °C for 24 h (Ribeiro et al., 2020). Finally, the diameters growth inhibition zones (mm) were registered using a millimeter scale. One-way ANOVA/Tukey *post hoc* statistical tests were used to evaluate intergroup statistical differences ($p < 0.05$), determined by Microsoft Excel Professional Plus 2019 and Action version 2.9 software.

2.3 Preparation of nanostructured lipid carriers

Different NLC formulations were prepared by the hot ultrasonication-emulsification method. The lipid phase of NLC was composed of ucuuba butter (solid lipid) and EO (liquid lipid) with a total lipid concentration of 10% (w/v) at a ratio of liquid lipids to total lipids of 0.1:1. So, the final EO concentration in NLC was 10 mg/mL, corresponding to 10% of the total lipid matrix of NLC. Briefly, in a water bath, the lipid phase was heated 10°C above the melting point of the solid lipid (ucuuba butter). Simultaneously, the aqueous phase containing 5% poloxamer was heated to the same temperature as the lipid phase. Then, the aqueous phase was added dropwise to the lipid phase, under stirring at 10,000 rpm for 2 min, in an Ultra-Turrax high pressure homogenizer (Ultra-Turrax® T18). The obtained microemulsion was immediately ultrasonicated for 20 min in a Vibracell tip sonicator (Sonics & Mat. Inc., Danbury, USA) operated at 500 W and 20 kHz, with alternating cycles (on/off) at 30 s. After this step, the formed nanoemulsion was immediately cooled to room temperature (25° C) in an ice bath to solidify the nanoparticles (Ribeiro et al., 2021). For control experiments, solid lipid nanoparticles (without EO addition) were prepared following the same procedure. For didactic purposes, this system was called NLC control in this work (Table 1).

2.4 Long-term physicochemical stability study

The nanoparticle size (nm), polydispersion index (PDI) and Zeta potential (mV) were analyzed by diluting the NLC formulations (1:1000 v/v) in deionized water through the dynamic light scattering (DLS) technique by Litesizer equipment (Anton Paar Instruments, Anton Paar, Berlin, Germany). The same parameters were monitored for 12 months at 25°C, in triplicate (Ribeiro et al., 2017). One-way ANOVA/Tukey *post hoc* statistical tests were used to evaluate intragroup statistical differences over time ($p < 0.05$), calculated by GraphPad Prism 8 software.

2.5 Evaluation of *in vitro* antibacterial activity

All prepared NLC were evaluated by microdilution method to determine the MIC. This experiment was performed in 96-well plates, in triplicate. Different strains of *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* isolated from humans were analyzed. Bacterial suspensions were prepared as previously detailed in the screening step. They were diluted in 96-well plate to a final concentration per well of 1×10^6 CFU·mL⁻¹. Dilutions of all formulations were added into 96-well plates to reach 0.1 mL final volume. The negative control was Mueller Hinton Broth without bacteria. The 96-well plate of each bacterial strain was incubated at 37°C for 24 h. After this period, the MIC of *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* was determined for each NLC. One-way ANOVA/Tukey *post hoc* statistical tests were adopted to evaluate inter-group statistical differences ($p < 0.05$), determined by Microsoft Excel Professional Plus 2019 and Action version 2.9 software.

TABLE 1 Composition and concentration of total lipids (TL), ucuuba butter (LS), essential oils (EO) and surfactant used in each NLC formulation.

NLC	TL (%)	LS (%)	EO (%)
F1	10	9	Cinnamon, 1%
F2	10	9	Oregano, 1%
F3	10	9	Pepper mint, 1%
F4	10	9	Tea tree, 1%
F5	10	9	-
F6	10	9	Cinnamon and Oregano, 1%
F7	10	9	Cinnamon and Pepper mint, 1%
F8	10	9	Cinnamon and Tea tree, 1%
F9	10	9	Oregano e Pepper mint, 1%
F10	10	9	Oregano e Tea tree, 1%
F11	10	9	Tea tree e Pepper mint, 1%

TL = LS + OE; All NLC were stabilized with poloxamer 5% (w/v).

2.6 Structural characterization

The structural characterization was performed by infrared spectroscopy measurement (FTIR-ATR), differential scanning calorimetry (DSC) and field emission scanning electron analyses (FE-SEM). In the FTIR-ATR technique, the spectral range was 650 to 4000 cm^{-1} , with resolution of 2 cm^{-1} . The DSC analyses were performed using a TA equipment, model Q20. Samples were added in sealed aluminum pans. Analyses were carried out in a nitrogen atmosphere, at a flow rate of 50 $\text{mL}/\text{min}^{-1}$, in the temperature range from 0 to 100°C, at a heating rate of 10°C/min. In order to elucidate the morphology of NLC, FE-SEM analyses was performed using Tescan brand, model VEGA 3 microscope, operating in high vacuum under a voltage of 20 kV. Briefly, a drop of each sample was adhered to a cover glass previously fixed in a stub. After, the stubs were subjected to sputtering procedure for 120 s at 30 kV. Then, the samples were visualized in different magnifications.

2.7 Thermal cycling stability study

The thermal stability was evaluated by 3 consecutive temperature cycles totaling 21 days of monitoring in a Climatic Chamber Stability (Solab, Piracicaba, Brazil). Each cycle corresponded to 7 days of NLC storage under different critical conditions, such as: 4 days at 40°C followed by 3 days at 15°C. At the end each cycle, nanoparticle size (nm), polydispersity index (PDI) and Zeta potential (mV) were measured, by DLS through Litesizer equipment (Anton Paar Instruments, Anton Paar, Berlin, Germany), in triplicate. One-way ANOVA/Tukey *post hoc* statistical tests were used to evaluate intragroup statistical differences over time ($p < 0.05$), calculated by GraphPad Prism 8 software.

2.8 *In vivo* nanotoxicity assay on chicken embryo model

The nanotoxicity of NLC formulation was evaluated through *in vivo* chicken embryo model (CE), according to the following parameters: viability (%) and weight (g) (Ribeiro et al., 2020). 80 eggs of *Gallus gallus*, lineage W-36, were used. Before analyses, the eggs were submitted to ovoscopy to ensure the viability of the embryos at 7 days of development. Then, they were weighted and divided into 10 CE groups ($n = 8$) as follow: negative control (NC), eggs treated with 0.85% saline; NLC control (without EO), cinnamon EO emulsified with poloxamer (both at the same concentration as the NLC formulation) and NLC/CAN. All treatments were administered on CAM of CE at different concentrations: 0.5, 0.3 and 0.1% (w/v). Then, the eggs were incubated in automatic incubator (Premium Ecológica®) at 37.5°C and 55% air humidity for 7 days. The CE mortality was daily observed to determine the viability (%). At 14 days of embryonic development, the eggs were weighted.

The weight changes of CE were calculated through the difference between the weight of the eggs before and after the treatments, according to the following equation:

$$aW = (ce \cdot ysW \times 50) \div ieW$$

where aW = egg weight adjusted to 50 g; ce.ysW = embryo or annexes weight on 14 day of embryonic develop; ieW = initial egg weight on 7 day of embryonic develop.

The One-way ANOVA/Tukey tests were used to elucidate the intergroup statistical differences, in terms of CE weight ($p < 0.05$). For the CE viability test, chi-square test was used followed by the difference between two proportions test ($p < 0.05$). GraphPad Prism 8 software was used for these analyses.

3 Results

3.1 Screening of vegetable and essential oils

A preliminary screening of 14 natural oils was carried out in order to select those with the best activity against those microbial strains, in comparison with polymyxin B (Table 2). It was observed that cinnamon and oregano EO have inhibited all bacterial species and strains tested. Cinnamon EO exhibited inhibition zones ranging from 21.0 to 37.5 mm while oregano EO was responsible for inhibition zones ranging from 9.5 to 39.5 mm. Polymyxin B exhibited an average inhibition zone of 14.1 mm, considering all strains of the bacterial species evaluated. The assay also showed that peppermint EO and tea tree EO were capable of inhibiting the bacterial growth, producing inhibition zones ranging from 10.0 to 33.0 mm and 10.5 to 20.0 mm, respectively. Then, these EO were nanoencapsulated as active and structural compound of NLC.

3.2 Long-term physicochemical stability study

Figure 1 showed the results of the long-term physicochemical stability monitoring of the investigated formulations. The control formulation (F5) exhibited an average particle size of 303.5 ± 46.3 nm statistically higher ($p < 0.05$) than the others NLC based-EO, with the highest fluctuation in particle size over time.

Among the samples, only F8 formulation showed no statistically significant changes ($p = 0.09$) over time. Mean values of PDI ranged from 0.11 to 0.23 among the systems containing EO in its structure, while NLC control had a mean value of 0.24. In this case, F1 ($p = 0.36$), F2 ($p = 0.45$), F3 ($p = 0.08$), F6 ($p = 0.05$) and F8 ($p = 0.22$) did not show significant changes over time. The average values of Zeta potential of F5 were -35.7 mV. Among the systems containing EO, these values ranged from -32.2 to -35.2 mV. In this parameter, all NLC showed statistically significant variations ($p < 0.05$) over time.

TABLE 2 Growth inhibition zone of different bacterial species treated with different EO.

MEAN DIAMETER OF BACTERIAL GROWTH INHIBITION ZONE (mm)								
EO	<i>A. baumannii</i> 65JF	<i>A. baumannii</i> OXA-23	<i>A. baumannii</i> POA4-28	<i>K. pneumoniae</i> ATCC700603	<i>K. pneumoniae</i> Bactray	<i>K. pneumoniae</i> Responsive	<i>P. aeruginosa</i> IMP-1	<i>P. aeruginosa</i> PAO-1
Rosemary	0.00±0.00	0.00±0.00	9.00±1.41	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.00±0.00
Star anise	9.50±0.70	0.00±0.00	14.00±0.00***	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Basil	14.50±2.12***	12.50±0.70***	24.00±1.41*	4.50±0.70	13.50±0.70***	9.00±0.00***	11.00±1.41**	0.00±0.00
Cinnamon	30.00±0.00*	37.50±3.53*	31.50±0.70*	30.50±0.70*	40.00±1.41*	31.00±1.41*	34.50±6.36*	21.00±1.41*
Citronella	10.00±0.00	0.00±0.00	13.00±1.41***	0.00±0.00	18.00±2.82***	0.00±0.00	18.50±2.12 **	0.00±0.00
Clove	12.50±2.12***	11.50±0.70***	19.00±1.41**	9.50±0.70	14.00±1.41***	15.00±0.00**	15.00±0.00**	0.00±0.00
Peppermint	13.50±0.70***	0.00±0.00	25.00±7.07*	0.00±0.00	26.00±1.41**	10.00±1.41***	33.00±4.24*	0.00±0.00
Tea tree	15.00±0.00***	14.00±2.82***	20.00±11.31**	10.50±0.70	13.00±1.41	13.00±0.00**	16.00±0.00**	0.00±0.00
Oregano	22.50±0.70**	26.50±4.94**	35.50±6.36*	20.50±0.70**	39.50±0.70*	30.00±0.00*	30.00±0.00*	9.50±0.70**
Salvia	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	17.00±1.41***	0.00±0.00	9.50±0.70***	6.00±1.41***
Polymyxin B	15.00±0.00***	12.50±2.12***	13.00±0.00***	15.00±0.00***	13.50±2.12***	15.00±0.00**	14.00±0.00**	15.00±0.00*

NOTE: Means in the same column followed by distinct asterisks differ statistically from each other $p < 0.05$ (One way ANOVA followed by Tukey test);

* Represents the highest values of means similar to each other;

** Represents the second highest values of similar means;

*** Represents the third highest values of similar means.

3.3 Evaluation of the *in vitro* antibacterial activity

All prepared NLC were submitted to the *in vitro* antimicrobial activity test against the different bacterial strains through MIC test. This assay determined that the formulations F1, F6, F7 and F8 showed antimicrobial activity against all bacterial species and strains evaluated. However, F1 formulation, composed of cinnamon EO, ucuuba butter and stabilized by poloxamer, was the most efficient system against *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* inhibiting their growth, in the lowest possible concentration (Table 3). Thus, F1 after 12 months of storage was submitted to MIC assay again, in order to elucidate if the antimicrobial activity was maintained over time. It was observed that this nanosystem maintained the activity against all bacterial species investigated. Moreover, MIC values after a year was higher than that observed at the beginning of the study (Table 3) with the exception of Bactray *K. pneumoniae*, Responsive *K. pneumoniae* and IMP-1 *P. aeruginosa* species.

3.4 Structural characterization

Structural characterization was performed for NLC/CAN (F1), NLC control and its excipients. In FTIR-ATR analysis (Figure 2), the cinnamon EO spectrum revealed intense absorption bands at 1672 cm^{-1} (aldehyde C=O stretch), 1628 cm^{-1} (alkene C=C stretch) and 1450 cm^{-1} (C-C in the aromatic ring). In the vibrational spectrum of ucuuba butter, there are characteristic bands centered at 2915 cm^{-1} and a group of bands between 1393 and 1177 cm^{-1} , corresponding to the $-\text{CH}_2$ vibration. Strong bands centered at

1732 and 2848 cm^{-1} related to the vibration C=O and O-CH₂, respectively, were also observed. The surfactant poloxamer showed typical bands to aliphatic chain ethers centered at 2880 and 1348 cm^{-1} , associated with vibrations $\nu\text{O-CH}_2$ and $\nu\text{O-C-O}$, respectively. In this same spectrum, it was evidenced characteristic bands of the symmetric deformation δCH_2 at 1470 cm^{-1} and the axial stretching of CO (νCO) at 1098 cm^{-1} .

FTIR-ATR spectra of NLC/CAN and NLC (control) revealed characteristic bands for these systems, resulting from the overlap of most of ucuuba butter and poloxamer bands, as expected. However, the NLC/CAN spectrum showed that the absorption band in the regions at 2852 - 2883 cm^{-1} became less intense when compared to the NLC spectrum in the region of 2850-2916 cm^{-1} . Also, in the NLC/CAN spectrum, the absorption bands at 1672, 1628 and 1450 cm^{-1} observed in cinnamon EO were not detected.

Calorimetric analyses are necessary to demonstrate thermodynamic changes of formulations in relation to their excipients. Figure 3 shows the thermograms of NLC control, NLC/CAN and all their excipients. DSC analysis showed that NLC/CAN presented a thermal profile compatible with its solid lipid matrix (ucuuba butter, 45.4°C), with the melting point of 47.1°C. Endothermic peaks corresponding to the melting point of poloxamer and NLC control was observed at 54.5 and 47.6°C, respectively.

FE-SEM images of NLC/CAN and NLC control showed that both formulations presented nanoparticles with spherical morphology and with well-defined contours, as expected for this type of system (Figure 4). In addition, the images revealed spherical monodisperse distributed nanoparticles, compatible with the morphology of NLC composed of synthetic and traditional lipids excipients (Ribeiro et al., 2016; Calixto et al., 2021; Castro et al., 2021; Moura et al., 2021).

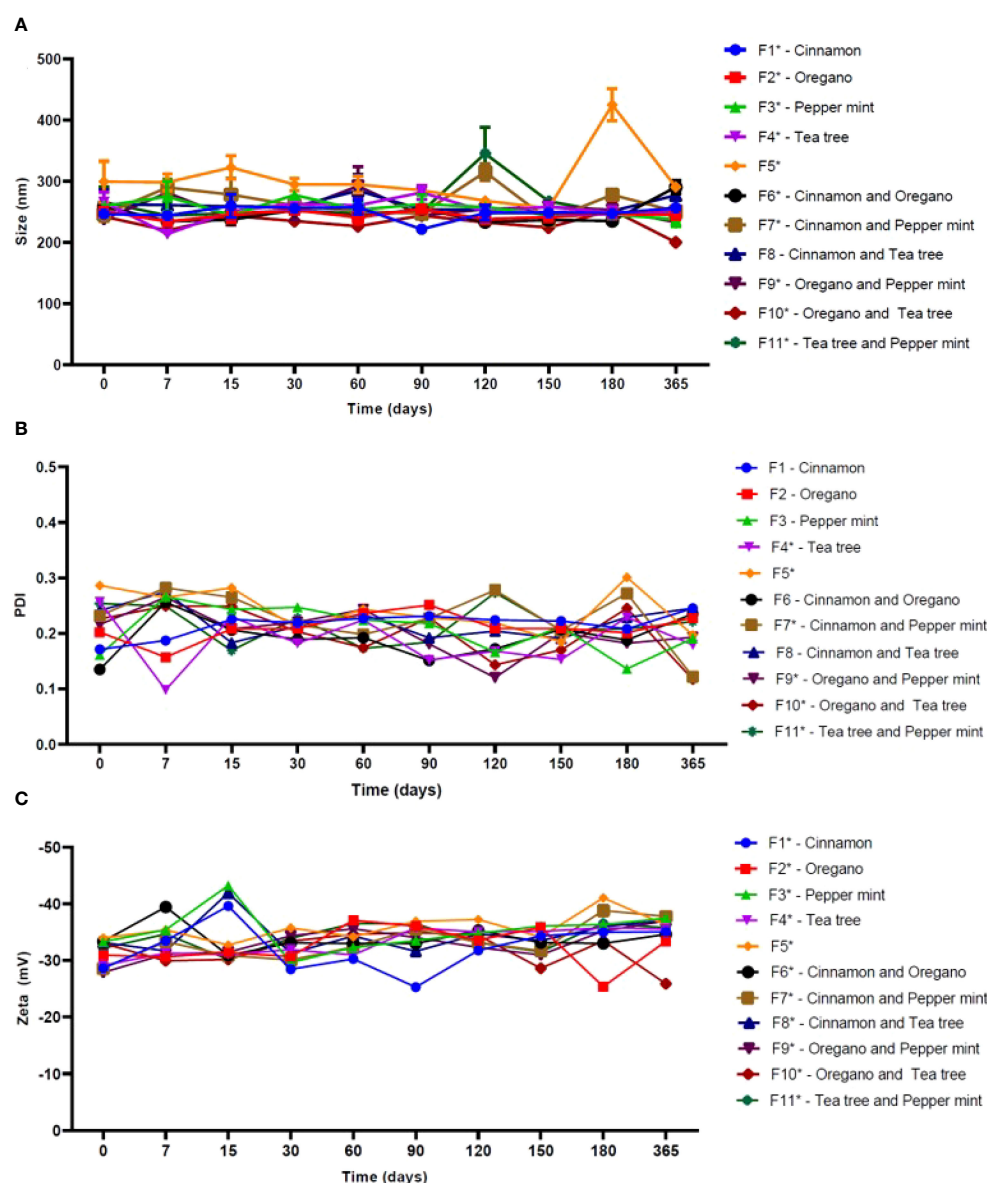


FIGURE 1

Physicochemical stability of NLC formulations in terms of size (A), PDI (B) and Zeta potential (C), over 365 days at 25°C (n = 3). NOTE: One-way ANOVA/Tukey were used to analyze intragroup statistics; *p < 0.05. PDI: polydispersion index.

3.5 Thermal cycling stability study

Thermal cycling stability study is useful to evaluate possible biophysical changes of nanoparticles under critical conditions of storage. Figure 5 showed a thermal cycling stability study of the NLC/CAN and NLC (control) formulations.

Particle size of NLC/CAN exhibited no statistically significant changes over time, ranging from 208.00 nm to 241.20 nm at the end of 21 days. The PDI values obtained guaranteed that NLC/CAN presented a monodisperse size distribution even after storage under critical temperature conditions, which did not show statistically significant changes. ZP values ranged from - 34.13 to - 39.00 mV for NLC/CAN.

3.6 *In vivo* nanotoxicity test through chicken embryo model

Figure 6 shows the mortality of embryos treated with negative control (NC = saline solution), NLC/CAN, NLC and cinnamon EO emulsified with poloxamer. There was no statistical difference between the treatments in comparison to the NC. Additionally, among alive embryos, there were no statistically significant macroscopic alterations compared to NC. However, regarding to the weight changes, the Figure 7 demonstrates that there was a statistically significant difference (p < 0.05) in the weight of embryos treated with EO emulsion in comparison to the NC.

TABLE 3 Minimum inhibitory concentration (mg/mL⁻¹) of NLC against different bacterial strains.

	Treatment mg/mL ⁻¹							
Species	After 2 months							After 12 months
strains	F1	F2	F6	F7	F8	F9	F10	F1
<i>A. baumannii</i>								
OXA-23	1.19 ± 0.00	> 5.00	3.33 ± 2.89	1.60 ± 2.78	3.33 ± 1.44	> 4.90	> 5.00	> 4.76
POA4-28	0.80 ± 0.34	1.25 ± 0.00	1.25 ± 0.00	1.20 ± 0.00	1.25 ± 0.00	3.27 ± 2.83	2.08 ± 0.72	1.19 ± 0.00
65JF	1.19 ± 0.00	> 5.00	5.00 ± 0.00	3.20 ± 1.39	2.50 ± 0.00	> 4.90	> 5.00	2.38 ± 0.00
<i>K. pneumoniae</i>								
ATCC700603	1.19 ± 0.00	> 5.00	5.00 ± 0.00	4.81 ± 0.00	5.00 ± 0.00	> 4.90	> 5.00	4.76 ± 0.00
Bactray	1.19 ± 0.00	> 5.00	2.50 ± 0.00	2.40 ± 0.00	2.50 ± 0.00	> 4.90	> 5.00	1.19 ± 0.00
Responsive	1.19 ± 0.00	> 5.00	2.50 ± 0.00	2.40 ± 2.41	2.50 ± 0.00	> 4.90	> 5.00	1.19 ± 0.00
<i>P. aeruginosa</i>								
IMP-1	1.19 ± 0.00	> 5.00	2.50 ± 0.00	2.40 ± 0.00	2.50 ± 0.00	> 4.90	> 5.00	1.19 ± 0.00
PAO-1	1.19 ± 0.00	> 5.00	5.00 ± 0.00	4.81 ± 0.00	4.17 ± 1.44	> 4.90	> 5.00	2.38 ± 0.00

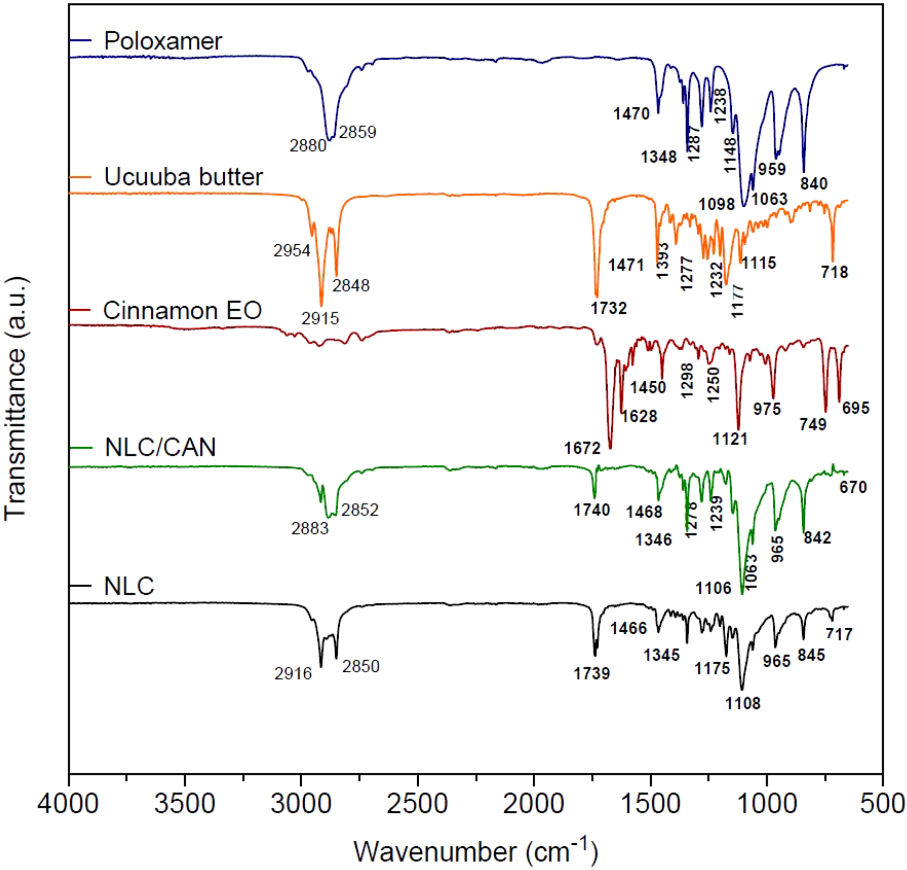


FIGURE 2 FTIR-ATR spectra in the region between 4000-500 cm⁻¹ of NLC/CAN, NLC and its excipients.

4 Discussion

Currently, there is a huge interest in the elucidation of the therapeutic properties of EO. There are several works that have described exciting results of EO uses in the cosmetic, agriculture, food and pharmaceutical fields (Castro et al., 2019; Guidotti-Takeuchi et al., 2022; Chukwuma et al., 2023).

The identification of bioactive compounds that represent an effective alternative to antibiotics is an important step in drug development in an attempt to minimize the bacterial resistance (Kaskatepe et al., 2016). In here, it was identified that cinnamon, oregano, peppermint and tea tree EO showed activity against the bacterial strains studied. However, the performance of oregano EO was strain-dependent in relation to *P. aeruginosa*, once a single strain showed resistance to this EO. Similar result was found for strains of *P. aeruginosa*, tested under the same conditions, showed different responses to the same EO (Coseriu et al., 2023), corroborating the difficulty to treat this type of infection. Other studies have also showed significant antibacterial properties for cinnamon EO, which inhibited the growth of *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* (El-Kattan and Allam, 2021; Ganić et al., 2022; Coseriu et al., 2023). Furthermore, this EO exhibited higher antibiofilm activity against *K. pneumoniae*, followed by peppermint EO (Sarwar et al., 2022). Man et al. (2019) investigated the antibacterial activity of some EO against some Gram-positive and Gram-negative bacteria, including *K. pneumoniae* strains. It was also observed that oregano EO is a bioactive compound with promising antibiotic activity.

NLC formulations were monitored in terms of particle size (nm), polydispersion index (PDI) and Zeta potential (mV) in the long-term stability study in order to ensure the shelf time of such systems. This study is mandatory to ensure the quality control of the formulations. Regarding the particle size, there was not observed any tendency to increase in size as a function of time, which would be indicative of some physicochemical instability process, such as agglutination of nanoparticles. Moreover, regarding the size

distribution profiles, a successful lipid nanocarriers should present homogeneous size distribution (monodisperse), with values as low as possible, to ensure long-term stability. Values ≤ 0.2 are usually accepted to describe a monodisperse distribution. Additionally, nanocarrier formulations with constant and narrow size distribution are correlated with better clinical results (Danaei et al., 2018). According to observed here, EO was responsible for reducing and making more uniform the particle size distribution of NLC when compared to the control formulation (without EO), elucidating its structural role in the system in addition to the already bioactivity claimed (Ribeiro et al., 2021). Furthermore, Zeta potential also brings information about the stability of colloidal aqueous dispersions (Souto et al., 2004). In general, good stability is achieved with Zeta potential values above ± 25 mV ensuring electrokinetic stabilization of the colloidal dispersion (Thatipamula et al., 2011). For sterically stabilized systems, as those use non-ionic as surfactants, the Zeta values are more related to the sample composition, presenting slightly or moderately negative values, as observed here (Scioli Montoto et al., 2020), indicating a desirable colloidal electrokinetic stability.

In addition, the thermal cycling study was carried out to bring some evidence regarding the control quality of NLC submitted to critical storage conditions. Such test provides information of the temperature and humidity conditions for a successful transport and distribution of NLC. In agreement with the long-term stability study. Such results strongly suggested that a cheap process to scale up, storage and distribution of such nanoformulations will be possible.

The *in vitro* antimicrobial activity of such formulations was determined by MIC. It was observed that the formulations that had cinnamon EO in their composition (F1, F6, F7 and F8) have showed activity against all the analysed strains/species and that the F1 formulation maintained *in vitro* antibacterial activity even after 1 year of storage at room temperature. Other work also revealed that the loaded cinnamon EO exhibited promising antibacterial activity and prevented both biofilm formation from *A. baumannii* strains,

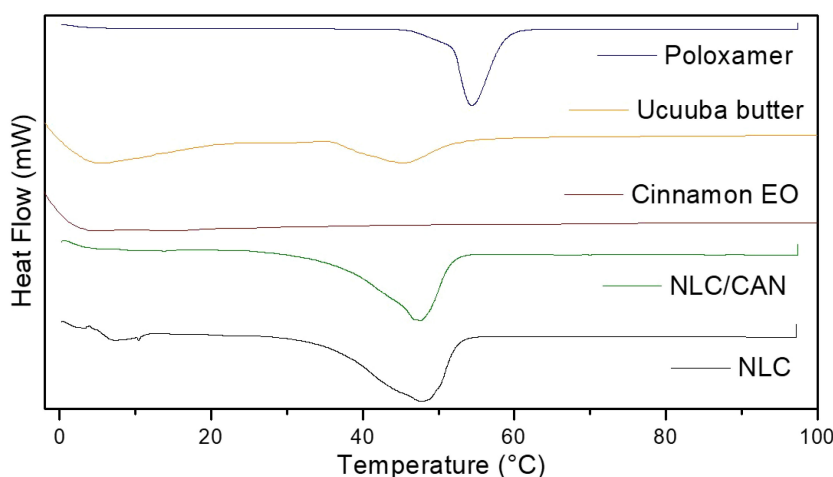


FIGURE 3
Thermogram obtained by DSC of NLC/CAN, NLC and its excipients.

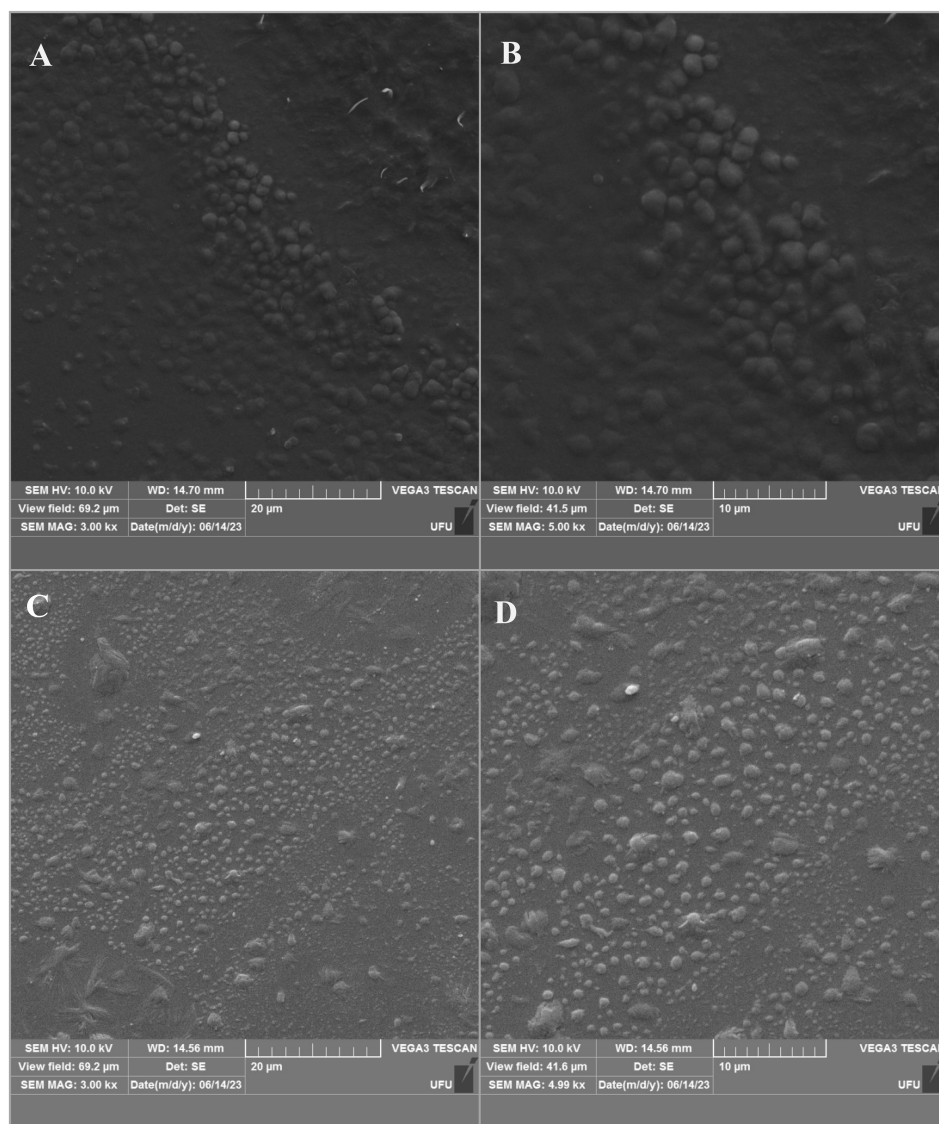


FIGURE 4

Scanning Electron Microscopy images of NLC/CAN (A, B) and NLC control (C, D). The images are available in two different magnifications: 3000x (left) and 5000x (right).

also exhibiting antibiofilm capacity (Ganić et al., 2022). Another work described that the strains of *P. aeruginosa* also showed susceptibility to cinnamon EO at lowest concentrations, (0.125% v/v). Such bactericidal effect of EO from the bark was higher than cinnamon leaf, probably due to the presence of Σ -cinnamaldehyde as the main component (85.3%). Furthermore, it was already shown that this EO induced rapid death of *P. aeruginosa* PAO-1, possibly due to the bacterial cell membrane rupture (Elcocks et al., 2020) and the synergistic action among its bioactive components, affecting gene expression and the activity of bacterial efflux pumps (Cosseriu et al., 2023).

Then, it was selected NLC/CAN (F1) as the most effective nanoformulation. However, it is still necessary to continue investigating the efficacy of this nanosystem in more complex biological models, in order to obtain more robust results.

The F1 structural characterization through FTIR-ATR, DSC, FE-SEM methods, evidenced that the excipients were compatible with each other. In general, the F1 spectrum was based on the overlapped bands of ucuuba butter and poloxamer, their major components. However, in the NLC/CAN spectrum, there is strong evidence of interaction with EO, due to the absence of absorption bands in the regions at 1672, 1628 and 1450 cm^{-1} of cinnamon EO, suggested an excellent miscibility between ucuuba butter and EO. Furthermore, NLC have showed thermal stability up to 100°C, without any thermal event related to the degradation of fatty acids, as required for this type of system. The structural and thermal analyses have also confirmed that the EO is part of the structure of the NLC, providing lower viscosity and crystallinity to the lipid matrix (Ribeiro et al., 2021). Such achieved corroborated the complex supramolecular arrangement of NLC in comparison with

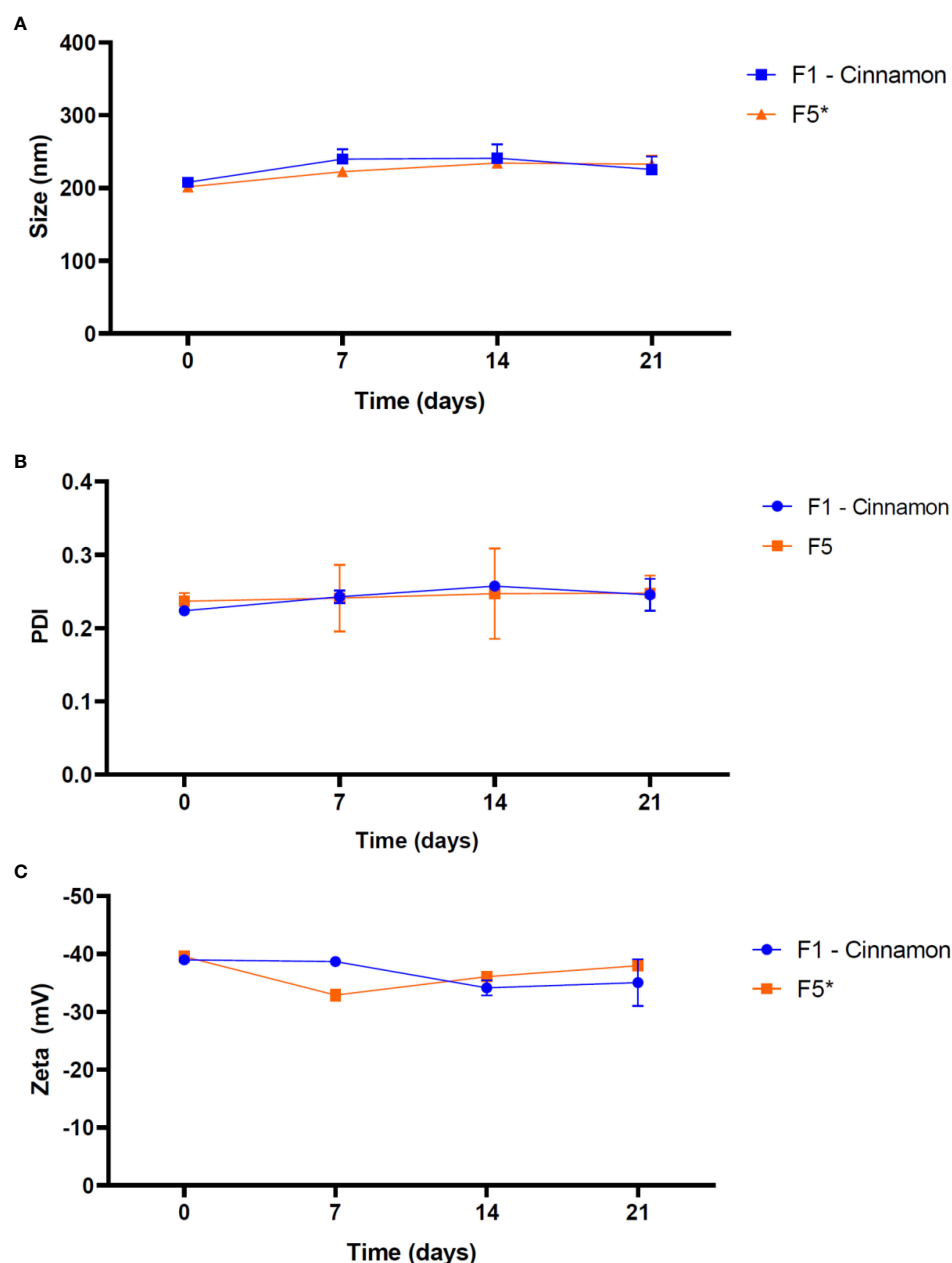


FIGURE 5

Thermal stability of NLC formulations in terms of size (A), PDI (B) and Zeta potential (C), at the end each temperature cycle. 1 cycle = 7 days of storage (4 days at 40°C followed by 3 days at 15°C). NOTE: One-way ANOVA/Tukey were used to analyze intragroup statistics; * $p < 0.05$. PDI: polydispersion index.

SLN (composed of only solid lipid in the structural matrix). The blend of lipids results in a more amorphous form with structural imperfections resulting in more stable and efficient formulations (Souto et al., 2004).

Finally, the safety of NLC/CAN formulation was ensured through the *in vivo* nanotoxicity test on CE model. All the nanoformulations and concentrations tested were safe, once they did not show any statistically significant differences in comparison to the NC, in all the parameters analysed. However, the CE weight changes treated with cinnamon EO emulsion was statistically significant different ($p < 0.05$) compared to the NC, confirming the protective effect of loading EO by NLC. It is worth mentioning that emulsion was the first colloidal delivery systems already

reported, and it is widely known that system is unstable, being not applicable as treatment candidate or nanosantizer (Araújo et al., 2019). This approach had also determined the higher safe dose on embryos for further efficacy tests. Moreover, it was observed that NLC/CAN, composed of cinnamon EO, ucuuba butter and poloxamer was safe up to 50 mg/mL of cinnamon EO loaded by NLC, much higher than the effective concentration value given by MIC, which was around 1.2 mg/mL.

The benefits of nanostructured drugs can be realized mainly for cancer treatments, with some nanocolloidal systems applied as gold standard treatments, as Doxil® (Ben Venue Laboratories, Inc Bedford, OH) and Myocet® (GP-Pharm, Barcelona, Spain). In the commercial antimicrobials nanoparticle-based drugs, 3 lipid-based

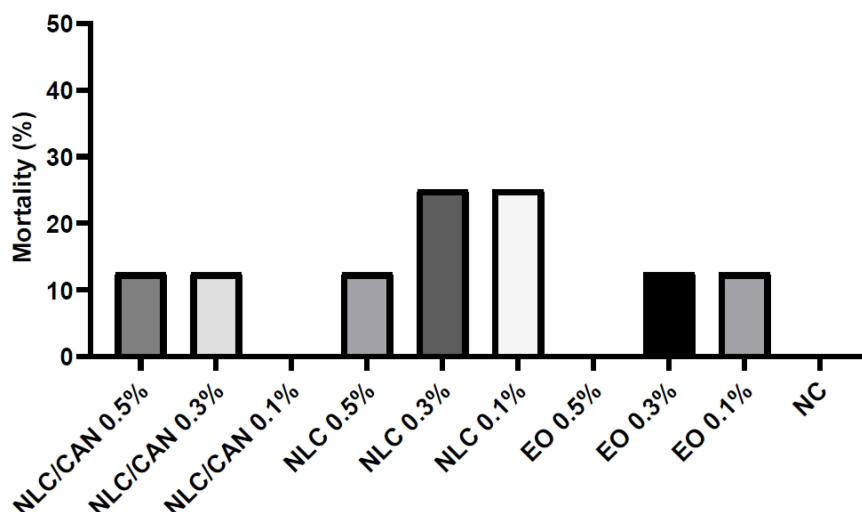


FIGURE 6

Mortality of chicken embryos treated with NLC/CAN, NLC and cinnamon EO emulsion (EO) at different concentrations.

formulations of amphotericin B are commonly used for the systemic treatment of fungal infections: Ambisome® (Gilead Sciences, Inc, San Dimas, CA), Amphotec® (Ben Venue Laboratories, Inc, Bedford, OH) and Abelcet® (Sigma-Tau PharmaSource, Inc, Indianapolis, IN) (Adler-Moore and Proffitt, 2002; Yeh et al., 2011). Despite the excellent biophysical and biological activity of nanoformulations, there are a lot of challenges to be overcome until they reach the market. This is

still necessary considering the particularities on nanostructured system in the regulatory issues, as well as in the biological assays planning, in order to decrease the time of approval and provided standardized methods to specifically evaluated the nanotoxicity in the biological tissues and environment.

The data found in here strongly suggest that NLC/CAN formulation remained stable up to 12 months, exhibiting excellent properties in terms of particle size, homogeneity and surface charge

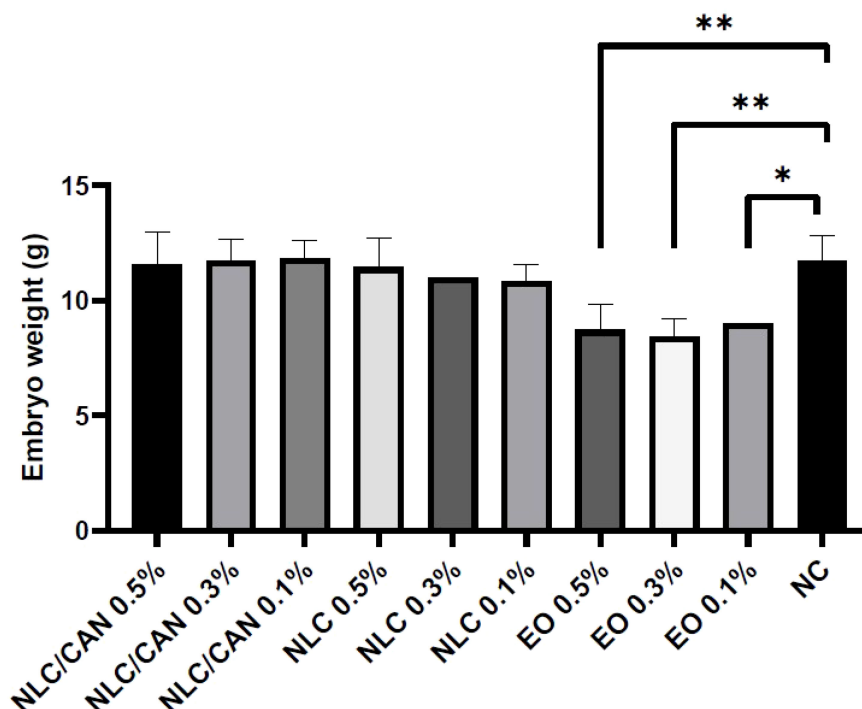


FIGURE 7

In vivo toxicity study in terms of chicken embryo weight under treatment with NLC/CAN, NLC and cinnamon EO emulsion (EO) at different concentrations. ** $p < 0.01$ and * $p < 0.03$.

at the end of experiment. So, the NLC/CAN is a promising system, being economically viable to be used as a surface disinfectant or antiseptic of hospitals. Besides, the same system has structural properties that allows its administration on parenteral and topical (skin and mucosa) routes, aiming to be a safe and more efficient antibiotic treatment against multidrug resistant species.

5 Conclusions

NLC formulations that encapsulated EO demonstrated excellent long-term stability (1 year, at 25°C). Such system was effective against *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolated from humans. The structural characterization confirmed a supramolecular organization compatible with NLC formulations and thermal stability of the systems up to 100°C, making them suitable for use in biomedical applications. The evaluation of nanotoxicity in embryos showed that the NLC/CAN formulation was safe under all conditions tested. Furthermore, it was observed that the safe concentration was higher than the effective one. Based on these promising results, the NLC/CAN will be further submitted *in vivo* efficacy studies on chicken embryo and mammal model, in order to find an effective, safe and low-cost therapeutic option against infections by multidrug-resistant bacteria.

Data availability statement

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

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

Ed: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. LB: Funding acquisition, Project administration, Resources, Supervision, Writing – review

& editing. BF: Methodology, Formal analysis, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. RR: Writing – review & editing. SS: Investigation, Writing – review & editing. HP: Investigation, Writing – review & editing. Fd: Investigation, Writing – review & editing. LR: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

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

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The effects and mechanisms of natural products on *Helicobacter pylori* eradication

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Helicobacter pylori (*H. pylori*) eradication is pivotal for alleviating gastric mucosal inflammation and preventing the progression of gastric diseases. While antibiotic-based therapies have achieved significant success in *H. pylori* eradication, challenges such as antibiotic resistance, drug toxicity, side effects, nonadherence, inapplicability, and disruption of gastrointestinal microflora have emerged. Updated therapies are urgently needed to suppress *H. pylori*. Nature has provided multitudinous therapeutic agents since ancient times. Natural products can be a potential therapy endowed with *H. pylori* eradication efficacy. We summarize the basic information, possible mechanisms, and the latest research progress of some representative natural products in *H. pylori* eradication, highlighting their safety, accessibility, efficiency, and ability to overcome limitations associated with antibiotic application. This review highlights the potential therapeutic advantages of incorporating ethnomedicine into anti-*H. pylori* regimens. The findings of this review may provide insights into the development of novel natural products and expand the therapeutic options available for *H. pylori* eradication.

KEYWORDS

Helicobacter pylori, eradication, antibiotic resistance, ethnomedicine, natural products, complementary therapy

1 Introduction

Helicobacter pylori (*H. pylori*) infection is a major cause of gastric mucosa injury. Approximately 1% of infected individuals will progress to gastric cancer (de Vries et al., 2008). Therefore, the WHO classified *H. pylori* as a class I carcinogen in 1994 (Cancer, 1994). According to statistics, approximately half of the world's population is affected by *H. pylori* infection, which accounts for 15% of the global cancer burden (Shah et al., 2021). Therefore, this pathogen exerts a profound influence on society and the economy, and it is crucial to find an effective method for *H. pylori* eradication to avoid potential accompanying diseases. Some risk factors have been reported to be associated with *H. pylori* infection, such as smoking,

unboiled water and uncooked food intake, poor socioeconomic conditions, and early childhood exposure to the bacterium (Leja et al., 2019; Yuan et al., 2022). Antibiotic combination therapy is needed to eradicate *H. pylori*, for example, the standard PPI-clarithromycin-containing triple therapy and bismuth quadruple therapies (Malfertheiner et al., 2017). However, in the context of a global increase in antibiotic consumption, antibiotic resistance of *H. pylori* has reached a crisis point, which poses a severe threat to the current regimens (Fallone et al., 2016). Consequently, clarithromycin-resistant strains of *H. pylori* were recognized by the WHO in 2017 as one of 12 priority pathogens in urgent need of novel antibiotics or alternatives (Tacconelli et al., 2018). As a result, new therapeutic therapies are imperative.

Complementary and alternative treatments with features of lower toxicity and low cost are attractive, and natural compounds have long been viewed as vital candidates for anti-*H. pylori* regimens. Researchers in gastroenterology or bacteriology domains have shown remarkable interest in the antimicrobial activities of natural products, especially for the possible lessened likelihood of developing genetic resistance. With a view to identifying more medicinal natural products and their synthetic variations, it is ideal to take a deep look into ethnomedicine, for example, traditional Chinese medicine (TCM). Numerous *in vitro* and *in vivo* studies have been conducted to investigate the therapeutic effect of ethnomedicine and their corresponding natural products against *H. pylori*. For instance, the *in vitro* efficacy of artemisone and artemisinin derivatives against *H. pylori* was demonstrated in the work by Sisto et al. (Sisto et al., 2016), while it also indicated a synergistic effect combined with standard drugs. In addition, Krzyżek et al. identified myricetin as a natural substance that hampers the morphological transition of *H. pylori* from spiral to coccoid forms and therefore increases susceptibility to antibiotics (Krzyżek et al., 2021). Confronting the antibiotic resistance affecting *H. pylori* eradication, such studies elucidate applicable natural products as alternative therapies. Great progress has been made in this field, but few natural product-based regimens have been recommended by clinical guidelines, and the

mechanisms behind ethnomedicine are still not clearly explained. Consequently, the keywords “*H. pylori*”, “natural products” and “ethnomedicine” were comprehensively searched in PubMed, Web of Science databases, China National Knowledge Infrastructure (CNKI), and Scopus databases. The publications were screened and those related to the theme of this review were included. Based on these relevant studies, we summarized the general situation and possible therapeutic mechanisms of natural products potentially with anti-*H. pylori* properties to serve as a reference for the development of novel drugs and expand therapeutic options against the bacterium (Figure 1).

2 Physiological properties and pathogenic mechanisms of *H. pylori*

H. pylori is a gram-negative, spiral, and microaerophilic bacterium that was discovered by Australian scientists Warren and Marshall in 1982 (Marshall and Warren, 1984). While the majority of bacteria are incapable of colonizing in such an acidic environment, *H. pylori* has evolved unique features that allow it to thrive in the exclusive ecological niche of the human stomach (Ansari and Yamaoka, 2019). These features include its mobility, helical shape, and ability to produce large amounts of urease, which neutralizes gastric hydrochloric acid by hydrolyzing urea into bicarbonate and ammonia (Solnick and Schauer, 2001). Furthermore, *H. pylori* possesses a chemotaxis system that enables it to detect pH gradients as well as sheathed polar flagella, which aid in reaching less acidic gastric mucosal surfaces (Roszczenko-Jasińska et al., 2020). Once there, adhesins such as blood-antigen binding protein A (BabA) facilitate the attachment of the bacteria to gastric epithelial cells (Sousa et al., 2022). In addition to factors involved in establishing infections, many clinical isolates of *H. pylori* produce virulence factors such as VacA, CagA, and cagPAI within the host cell cytoplasm. These factors play significant roles in immune evasion and disease induction (Sukri et al., 2020). Infected gastric tissues also exhibit elevated levels of reactive oxygen

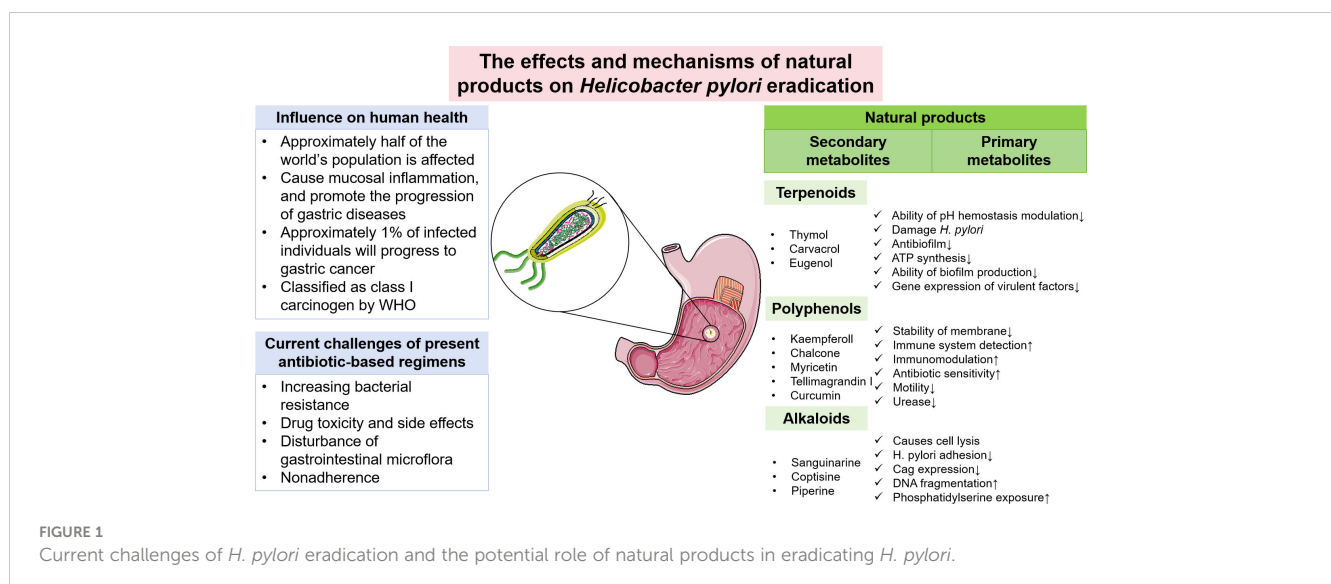


FIGURE 1

Current challenges of *H. pylori* eradication and the potential role of natural products in eradicating *H. pylori*.

species (ROS), leading to gastric inflammation with the production of various mediators (Kang and Kim, 2017).

3 General status, treatment guidelines, and current challenges of *H. pylori* eradication

H. pylori eradication is reported to help alleviate mucosal inflammation, restore normal mechanisms governing acid secretion, and stop the progression of gastric diseases (Sugano et al., 2015). Accordingly, the present consensus among all major gastroenterological societies is that *H. pylori* infection should be eradicated in individuals who tested positive unless there are compelling reasons (Sugano et al., 2015; Matsumoto et al., 2019; Shah et al., 2021). Since the microorganism was discovered, a wide variety of therapeutic strategies have been proposed to tackle *H. pylori*, such as clarithromycin triple therapy, bismuth or nonbismuth-based quadruple therapy, and rifabutin-based triple therapy (Cardos et al., 2021).

The option of eradication regimes should be determined according to the geographical area, in the context of regional differences in antibiotic resistance patterns, as well as the different common host genotypes of drug-metabolizing enzymes of the local population. In regions with low clarithromycin resistance (<15%), the Maastricht consensus report still endorses standard triple therapy as the primary treatment option (Malfertheiner et al., 2017), which consists of a proton pump inhibitor (PPI), amoxicillin, and clarithromycin or metronidazole. However, the regime is inapplicable in high clarithromycin resistance regions without preceding antimicrobial susceptibility testing. Metronidazole is an ideal substitute for clarithromycin in regions with low to intermediate metronidazole resistance rates (Malfertheiner et al., 2017). When high resistance to both antibiotics is observed, bismuth quadruple or nonbismuth quadruple, concomitant (PPI, amoxicillin, clarithromycin, and nitroimidazole) therapies are favorable options instead (Sugano et al., 2015; Malfertheiner et al., 2017).

Despite the significant progress made by antibiotic-based therapies, the present regimens are faced with a series of challenges, for example, high resistance to antibiotics, nonadherence, drug toxicity and side effects, and disturbance of gastrointestinal microflora (Matsumoto et al., 2019).

Increasing bacterial resistance to commonly used antimicrobial agents is one of the most common reasons for eradication failure, which has reached alarming levels worldwide and severely threatens the efficacy of treatment. Based on a systematic review and meta-analysis involving 65 countries, the incidence of primary and secondary resistance to clarithromycin, metronidazole, and levofloxacin is greater than 15% in most WHO regions (Savoldi et al., 2018). This threshold is commonly used to select alternative treatment regimens (Savoldi et al., 2018). Likewise, a recent prospective study covering 18 European countries also showed alarming *H. pylori* resistance to commonly used antibiotics and revealed a positive correlation between macrolide and quinolone consumption and corresponding *H. pylori* resistance

(Megraud et al., 2021). Accordingly, the last few decades have witnessed the unacceptable fact that *H. pylori* eradication rates of standard triple therapies experienced a sharp decrease from 80–90% in the 1990s to below 70%, and other regimens also encounter similar hurdles (O'Morain et al., 2018). To maximize the eradication rate and ensure the prudent use of antibiotics, individual antibiotic susceptibility testing seems to be a feasible solution, which is especially recommended in cases needing salvage therapies (O'Morain et al., 2018). Moreover, it is beneficial to collect regional antibiotic consumption data and clinical outcomes to provide local resistance reports and update guidelines for infection (Matsumoto et al., 2019).

In addition to the emergence of antibiotic-resistant strains, the possible adverse effects and nonadherence of some patients also impose restrictions on the utilization of conventional antibiotic-based eradication therapies. Antibiotics, especially at high dosages, may induce many side effects. For example, antibiotics are known to affect the balance of gut microbiota through both direct and indirect mechanisms. While eradicating targeted pathogens, antibiotics also discriminately kill or inhibit subsets of commensal microbes or disrupt the homeostasis of the symbiosis and codependency relationships among different subsets of gut microbiota (Zhang et al., 2019). In addition, some individuals may experience adverse reactions, including nausea, allergic reactions to antibiotics, and severe complications (liver and/or kidney dysfunction) (Takeuchi et al., 2014). The level of patient compliance is another crucial contributing factor for treating the infection, which can be influenced by barriers such as physical intolerance of medication, lack of understanding of the prescription, and high pill burden (Shah et al., 2021). The threshold of adherence for successful eradication varies depending on individual factors, but it was demonstrated that patients need to hold to at least >60% to >90% of the prescribed course to guarantee the eradication rate (Shah et al., 2021). Furthermore, host genetic polymorphisms of drug-metabolizing enzymes also account for the eradication failure of *H. pylori* infection. The metabolism-enhancing phenotypes of CYP2C19 are responsible for the rapid metabolism of some PPIs and thus influence the reduction in intragastric acidity. The optimal intragastric pH for *H. pylori* replication is between 6 and 8; thus, alkalizing the gastric environment can increase susceptibility to antibiotics in populations with metabolism-enhancing phenotypes of CYP2C19 (Safavi et al., 2015; Shah et al., 2021).

Due to the current persistence and rise of antibiotic resistance and other restrictions on current therapies, traditional antibiotic-based therapies have been severely weakened, and the development of novel and more effective antimicrobial compounds and novel strategies is drawing increasing attention worldwide (Matsumoto et al., 2019). However, owing to the complex biology of the pathogen, the high cost of research and the lack of financial support, the discovery and development of new therapeutics is not without challenges. To reduce the cost of developing new candidates for *H. pylori* eradication, it is beneficial to attach importance to the exploitation of natural product libraries and conduct thorough ethnobotanical and ethnomedical analyses, which may offer valuable inspiration for new treatments.

4 Natural products effects in *H. pylori* eradication and their healing mechanisms

4.1 The role of natural products and ethnomedicine

In light of the challenges faced by currently used *H. pylori* antibiotic-based eradication therapies, research on alternative treatment approaches is gaining popularity. With vast resources, nature has provided multitudinous therapeutic agents since ancient times and is seen as a promising source in the search for new compounds endowed with anti-*H. pylori* potential.

Ethnomedicine is the study of naturally obtained drugs based on traditional knowledge and practices of various ethnic groups (Lai et al., 2022), usually supported by ancient medical classics or passed down orally over generations. Generally, acquired from empirical observations and beliefs of indigenous peoples, the theoretical systems of ethnomedicine differ from scientific medicine, and some are still currently inexplicable by science. Nevertheless, ethnomedicine has been indispensable in health maintenance in households for centuries and is still widely used for primary health needs in extensive regions (Buenz et al., 2018).

Natural products are a series of bioactive chemical compounds produced by living organisms from nature, usually serving as the primary and secondary metabolites in biochemical pathways (Katz and Baltz, 2016). Typical examples of natural products include alkaloids, flavonoids, saponins, terpenes, etc. Although not universally recognized, natural products are widely acknowledged as the active components of numerous ethnomedicines, and the pertinent cutting-edge research findings have input vigor into the domain of drug discovery and development.

Several natural products and ethnomedicine have demonstrated antimicrobial activity through diverse scientific research as well as multiple generations of medical practice. Even before the discovery of *H. pylori*, a wide range of plants and substances have been employed to address gastric symptoms that are currently considered to be associated with the infection of this pathogen (Takagi and Harada, 1969; Escobedo-Hinojosa et al., 2012). Today, with a more in-depth knowledge of *H. pylori* after decades of exploration, ethnomedicine is still being used as a guide for the search for effective natural products and treatment approaches to cope with the infection (Vale and Oleastro, 2014), and some patent medicines have already been recommended as supplements in clinical guidelines. For example, traditional Chinese medicine and Kampo are recommended as treatment options by countries with prevalent use of traditional medicine (Helicobacter pylori Study Group, 2022; Miwa et al., 2022). The bioactivity of some natural products in inhibition of *H. pylori* has been summarized in Table 1.

4.2 The mechanisms of natural products in *H. pylori* eradication

Natural products are usually divided into two categories: primary metabolites and secondary metabolites. Including carbohydrates,

lipids, proteins, and nucleic acids, primary metabolites are indispensable and directly involved in the life cycle of organisms (Chaturvedi and Gupta, 2021). Few of them, however, demonstrate pharmacologic actions against microorganisms. Several polysaccharides have exhibited antiadhesive properties against *H. pylori*, likely by inhibiting the crucial docking process of adhesins of *H. pylori* and gastric epithelium (Gottesmann et al., 2020).

In contrast, secondary metabolites are compounds that are not needed for life, usually not only possessing a wide range of biological functions but also having special mechanisms of action (Li et al., 2021). The scientific community has invested significant expectations in the utilization of secondary metabolites, and a large number of studies have been performed to reveal the pharmacological value of these compounds. We summarized the basic information and possible mechanisms of some representative secondary metabolites for *H. pylori* eradication (Table 2 and Figure 2). The section below describes the latest research progress of secondary metabolites for *H. pylori* eradication.

4.2.1 Terpenoids

Terpenes are a large class of plant secondary metabolites formed with five-carbon isoprene (C_5H_8), and classifications of terpenoids are based on the number of isoprene units in their structure (Khan et al., 2018). As widely diffused chemicals in plants, many types of terpenoids have been shown to have anti-*H. pylori* properties, and here, we will update the reports of the antimicrobial effects of some terpenoids.

Monoterpenoids are a series of natural products that possess two isoprenes in their molecules, which are the prevailing components of the essential oils of pine, lemon, thyme, tea tree, etc., and many of them have demonstrated gastroprotective activity *in vivo* as well as antimicrobial properties *in vitro* (Rozza et al., 2011). Typical examples with anti-*H. pylori* properties include limonene and β -pinene, which are predominant components in *Citrus lemon* (Rutaceae) essential oil and have been demonstrated to exert antibacterial effects *in vitro*. Limonene was shown to elevate mucus secretion by ensuring adequate PGE_2 levels, thus limiting the colonization of *H. pylori* and isolating gastric mucosa from ulcerative factors (Moraes et al., 2009). Additionally, monoterpenes demonstrate anti-inflammatory capacity by regulating oxidative stress and the inflammatory response of gastric mucus. Consequently, the expression of MPO, NF- κ B, and proinflammatory cytokines such as TNF- α , IL-6, and IL-1 β is decreased, while that of IL-10 is increased (de Souza et al., 2019). β -Pinene can also promote mucus production, relieve oxidative stress and inflammation and inhibit *Nf- κ b* expression to exert its gastroprotective properties (de Souza et al., 2019). Similarly, α -pinene manifests gastroprotective properties with modulation of oxidative stress and PGE_2 and histamine levels *in vivo* (Al-Sayed et al., 2021). β -Myrcene also possesses the capacity to block the growth of *H. pylori*, and the corresponding MIC of the monoterpene is 500 μ g/mL (Wei and Shibamoto, 2010). For gastroprotective effects, β -myrcene prevents gastric damage by the marked upregulation of antioxidant enzyme activity, with decreased activity of superoxide dismutase (SOD) and increased levels of glutathione peroxidase (GPx), glutathione reductase (GR), and total glutathione (Bonamin et al., 2014). Moreover, terpenoid phenols, thymol and carvacrol, are reported

TABLE 1 Summarization of the bioactivities of natural products in *H. pylori* inhibition.

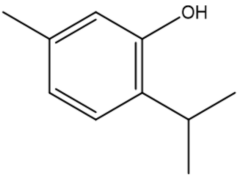
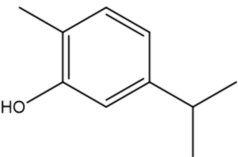
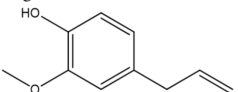
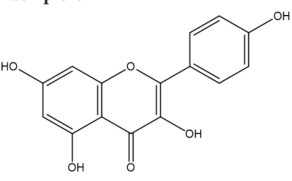
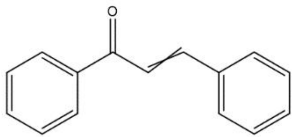
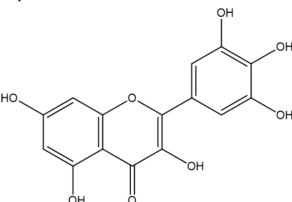
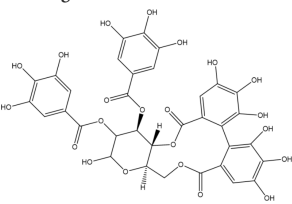
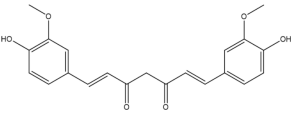
Compounds name	MIC (μg/mL)	MBC (μg/mL)	<i>H. pylori</i> strains	Reference
Limonene	75	/	ATCC 43504	(Rozza et al., 2011)
	199.2	/	ATCC 43504	(Villa-Ruano et al., 2018)
β-pinene	500	/	HATCC 43504	(Rozza et al., 2011)
	296.9	/	ATCC 43504	(Villa-Ruano et al., 2018)
α-pinene	/	100	P1	(Bergonzelli et al., 2003)
β-Myrcene	500	/	ATCC 43504	(Bonamin et al., 2014)
Thymol	64-128	/	Eight strains of <i>H. pylori</i> (one reference and seven clinical isolates)	(Sisto et al., 2021)
Carvacrol	16–64	32–64	Nine strains of <i>H. pylori</i> (one reference strain and eight clinical isolates)	(Sisto et al., 2020)
Eugenol	23.0-51.0	/	<i>H. pylori</i> isolates and standard strain NCTC 11637	(Elbestawy et al., 2023)
Glycyrrhetic acid	50	/	29 different <i>H. pylori</i> strains	(Krausse et al., 2004)
Kaempferol	>600	/	ATCC 43504 and 26695	(Escandón et al., 2016)
Chalcone (Xinjiachalcone A)	12.5-50	/	Seventeen <i>H. pylori</i> strains including clinical ones	(Bodet et al., 2014)
Myricetin	160	320	ATCC 700824, 51932	(Krzyżek et al., 2021)
Taxifolin	625	/	ATCC 43629	(Stenger Moura et al., 2021)
Tellimagrandin I	12.5	/	NCTC 11638, ATCC 43504, A-13, A-19	(Funatogawa et al., 2004)
Tellimagrandin II	6.25-12.5	/	NCTC 11638, ATCC 43504, A-13, A-19	(Funatogawa et al., 2004)
Curcumin	30	40	ATCC 700392	(Darmani et al., 2020)
Berberine	25	/	NCTC 26695 and SS1	(Wu et al., 2023)
Coptisine	16-32	32-64	ATCC 43504,700392 and PMSS1	(Tang et al., 2023)
Palmatine	50	/	NCTC 26695 and SS1	(Wu et al., 2023)
	32-64	64-128	ATCC 43504,700392 and PMSS1	(Tang et al., 2023)
Sanguinarine	6.25–50	/	A total of 14 clinical isolates	(Mahady et al., 2003)
Piperine	125	/	60190, NTCC 11637 and Tx30a	(Tharmalingam et al., 2014)

antimicrobial agents with MIC ranges of 64-128 μg/mL and 16-64 μg/mL (Grande et al., 2021). A specific concentration of carvacrol or thymol is observed to cause an increase in membrane fluidity, leakage of essential ions, and limited ATP synthesis in pathogens, which is partly due to the hydrophobic character and may also be attributed to the presence of a hydroxyl group and a system of delocalized electrons in their structures, causing a decrease in the membrane pH gradient and thus interfering with ATP synthesis (Ultee et al., 2002). Additionally, the inhibition of carbonic anhydrase (CA), a series of enzymes that catalyze the equilibrium between CO₂ hydration, may also play a crucial role in the antibacterial mechanism of the two terpenoids. *H. pylori* CAs are pivotal in the modulation of pH hemostasis, the integrity of the bacterial membrane, and the ability to produce biofilms, which are promising druggable targets in eliminating pathogenic microorganisms (Grande et al., 2021). Last, Eugenol is thought to be one of the most active natural products against *H. pylori*, with MICs ranging from 23.0 to 51.0 μg/mL (Elbestawy et al.,

2023). Previous studies indicate that eugenol possesses antibiofilm activities against *H. pylori* and can downregulate the expression of virulence factors, while eugenol can also relieve *H. pylori*-related gastritis by its anti-inflammatory activities (Hu et al., 2018).

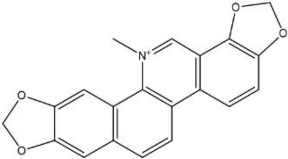
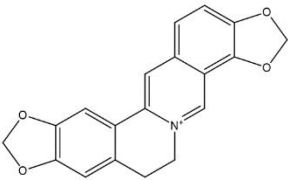
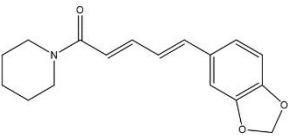
Sesquiterpenoids are another group of predominant secondary metabolites in various plant essential oils. For instance, over 70% of the components present in cedarwood essential oil are sesquiterpenoids, including α-, β-cedrene, thujopsene, cedrol, and cuparene, and the compound group displayed excellent efficacy in inhibiting urease activity and *H. pylori* growth (Korona-Glowniak et al., 2020), which may indicate that the anti-*H. pylori* activity of sesquiterpenoids. Additionally, with a similar structure to the major components in cedarwood essential oil, patchouli alcohol, a tricyclic sesquiterpenoid, exhibited antimicrobial properties toward *Streptococcus mutans* via the inhibition of DNA polymerase in a previous study (Takao et al., 2012), which may also offer a clue for the anti-*H. pylori* activity of sesquiterpenoids.

TABLE 2 Summarization of the basic information and possible mechanisms of some representative secondary metabolite for *H. pylori* eradication.

Compounds name	Reported from (plant name)	Mechanisms	Reference
Terpenoids			
Thymol 	<i>Thymus kotschyanus</i>	Ability of pH hemostasis modulation↓; damage <i>H. pylori</i> ; membrane integrity; ATP synthesis↓; ability of biofilm production↓.	(Grande et al., 2021; Sisto et al., 2021)
carvacrol 	<i>Origanum vulgare</i>	Ability of pH hemostasis modulation↓; damage <i>H. pylori</i> ; membrane integrity; ATP synthesis↓; ability of biofilm production↓.	(Oliveira et al., 2012; Sisto et al., 2020; Grande et al., 2021)
Eugenol 	<i>Eugenia caryophyllis</i>	Antibiofilm↓; gene expression of virulent factors↓;	(Wei and Shibamoto, 2010; Hu et al., 2018; Elbestawy et al., 2023)
Polyphenols			
kaempferol 	<i>kaempferol galanga</i>	Stability of membrane↓.	(Yang et al., 2022)
chalcone 	species of the <i>Leguminosae</i> , <i>Asteraceae</i> and <i>Moraceae</i> families	Interactions between <i>H. pylori</i> and gastric epithelium↓; motility↓; urease↓;	(Michalkova et al., 2023)
myricetin 	<i>Myrica</i>	Immune system detection↑; antibiotic sensitivity↑; biofilm↓.	(Krzyżek et al., 2021)
Tellimagrandin I 	<i>Cornus canadensis</i>	damage <i>H. pylori</i> ; membrane integrity;	(Funatogawa et al., 2004)
Curcumin 	<i>Turmeric</i>	Urease↓; immunomodulation↑.	(De et al., 2009; Khonche et al., 2016; Judaki et al., 2017; Shetty et al., 2021)

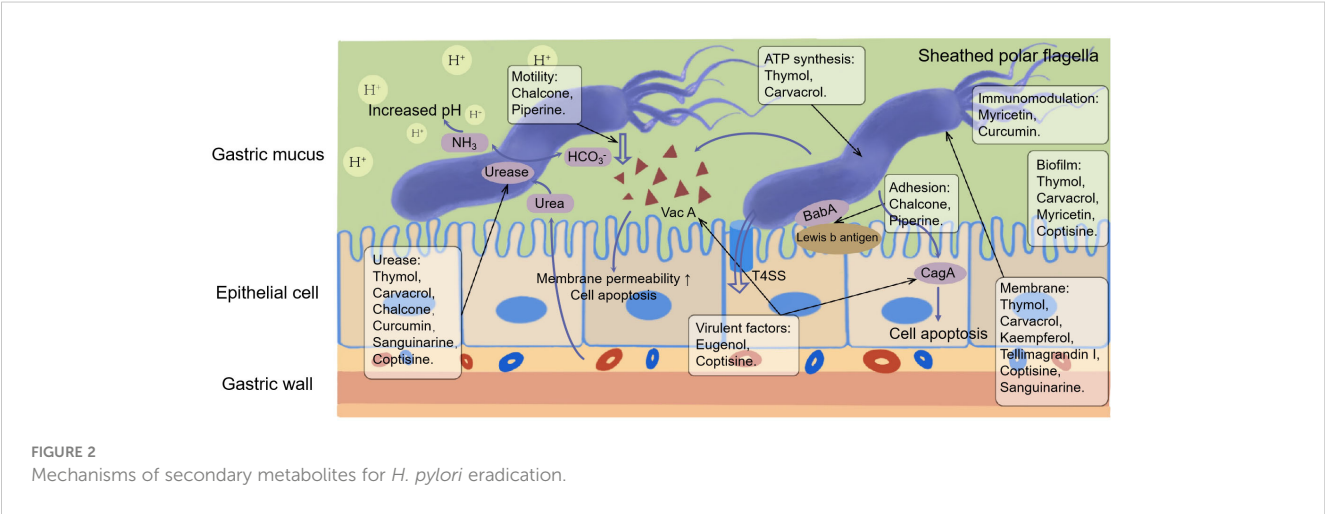
(Continued)

TABLE 2 Continued

Compounds name	Reported from (plant name)	Mechanisms	Reference
Alkaloids			
sanguinarine 	<i>Zanthoxylum nitidum</i>	Urease↓; cell lysis.	(Thawabteh et al., 2019; Lu et al., 2022)
coptisine 	<i>Rhizoma Coptidis</i>	Urease↓; Cag expression↓; DNA fragmentation↑; phosphatidylserine exposure↑; membrane stability↓.	(He et al., 2022b; Tang et al., 2023)
Piperine 	<i>black pepper</i>	Motility↓; <i>H. pylori</i> adhesion↓.	(Tharmalingam et al., 2014)

Similarly, triterpenoids have also gained attention for their various biological functions, including anti-inflammatory, antitumor, antiviral and antimicrobial activities (Dzubak et al., 2006). Triterpene glycosides (saponin) are good examples of anti-*H. pylori* activity of triterpenoids. For instance, glycyrrhizic acid is a representative triterpenoid saponin enriched in *Glycyrrhiza glabra*, whose major metabolite, glycyrrhetic acid, was reported to exhibit rapid anti-*H. pylori* property *in vitro* (Wittschier et al., 2009). Glycyrrhetic acid is reportedly in possession of cytotoxic effects and can impair *H. pylori* growth. Nevertheless, some studies have indicated that long-term intake of saponin may lead to an increased risk of gastric lesions (Périco et al., 2015). Therefore, the use of saponin-rich plants as an antiulcer ethnomedicine should be under careful consideration.

Tetraterpenoids are terpenoids that consist of a C40 structure, and carotenoids are a well-known subclass of tetraterpenoids that possess potent antioxidant properties. Previous research has explored the potential of some carotenoids in the treatment of *H. pylori*-related gastric diseases, including β-carotene (Bae et al., 2021), astaxanthin (Davinelli et al., 2019; Kim and Kim, 2021; Lee et al., 2022), and lycopene (Jang et al., 2012; Kim et al., 2018a). Carotenoids can be divided into two groups by the presence of oxygen, namely, xanthophylls and carotenes, and here, we will present β-carotene and astaxanthin as representatives. Abundant in orange-colored fruits and vegetables, β-carotene is a well-known carotenoid with marked antioxidant capacity, which may be structurally ascribed to the numerous conjugated double bonds (Kang and Kim, 2017).



β -carotene intake can suppress NADPH oxidase and stimulate antioxidant enzyme activity, thus contributing to the suppression of *H. pylori*-induced ROS generation as well as the level of iNOS and COX-2 expression. As a result, β -carotene exerts its anti-inflammatory effects by suppressing ROS-mediated inflammatory signaling (including MAPKs and NF- κ B), which functions in the prevention of inflammatory damage (Jang et al., 2009; Park et al., 2019; Bae et al., 2021). However, previous studies have mainly focused on the ability of β -carotene to modulate gastric carcinogenesis, and existing evidence has not shown explicit antimicrobial activity of β -carotene against *H. pylori*, which needs further exploration. In addition, astaxanthin is a xanthophyll carotenoid commonly found in crustaceans such as shrimp, crabs, and lobster (Davinelli et al., 2019), which is reported to have 10-fold stronger antioxidant activity than β -carotene. *In vivo*, a study using BALB/cA mice showed that an astaxanthin-rich algal meal could inhibit the colonization of *H. pylori* and suppress inflammation in gastric tissues of *H. pylori*-infected mice (Bennedsen et al., 2000; Naguib, 2000; Wang et al., 2000). Similarly, astaxanthin demonstrates its ability to prevent oxidative stress-mediated inflammation through ROS reduction (Kim et al., 2018b). Some research also illustrated that astaxanthin could induce a shift in the Th1/Th2 response pattern, thus enhancing the clearance of *H. pylori* (Davinelli et al., 2019). Additionally, a significant ability to inhibit H^+ , K^+ -ATPase was observed in astaxanthin esters, indicating a possible mechanism of *H. pylori* inhibition and a potent future of astaxanthin modification (Kamath et al., 2008).

4.2.2 Polyphenols

Polyphenols are another ubiquitous spectrum of natural products abundant in green tea, propolis, cranberry, etc (Gao et al., 2021; Ngan et al., 2021; Widelski et al., 2022), and can be grouped into classes such as flavonoids and tannoids. Structurally, polyphenols possess several hydroxyl groups on aromatic rings, which contribute to their antioxidant properties to reduce ROS production (Bonacorsi et al., 2012). Several investigations into the mechanisms of anti-*H. pylori* capacities have shown that polyphenols may lead to a decrease in urease activity, inhibition of cytotoxic activity as well as its binding with the gastric mucosa, and the rupture of outer membrane (Burger et al., 2002; Lin et al., 2005; Yahiro et al., 2005; Nohynek et al., 2006). According to a previous study, polyphenols as an adjuvant in the treatment of *H. pylori* infection can significantly improve the eradication rate (Wang et al., 2023; Wu et al., 2023), while no evidence has been found to suggest an increased risk of side effects. Consequently, polyphenols are suggested as an alternative treatment approach for *H. pylori* infection.

Among the polyphenol family, flavonoids are one of the most important and vast groups of compounds with a basic skeleton comprising two benzene rings linked through a heterocyclic ring, involving over 9000 species of molecules (Gonzalez et al., 2021). Many flavonoids have been shown to possess promising antimicrobial capacity against *H. pylori*, including kaempferol, chalcone, myricetin, taxifolin, and other compounds with proven validity (Krzyżek et al., 2021; Stenger Moura et al., 2021; Yang et al.,

2022; Michalkova et al., 2023). Flavonoids contribute to several antibacterial mechanisms, such as the inhibition of crucial enzymes for colonization, survival, and reproduction (Asha et al., 2013; Steinmann et al., 2013; Egas et al., 2018), and interference with the fluidity and stability of the cytoplasmic membrane (Tsuchiya and Iinuma, 2000; Yang et al., 2022). In addition, flavonoids can engage in the modulation of several intracellular pathways, such as MAPK and NF- κ B, thus attenuating the level of pro-inflammatory cytokines induced by *H. pylori* infection and improving gastric inflammation status (Wang and Huang, 2013). In addition to their antimicrobial and anti-inflammatory effects, flavonoids are capable of acting synergistically with antibiotics commonly utilized in the treatment of *H. pylori* infection (Krzyżek et al., 2021).

Tannoids (or tannins) are naturally occurring plant polyphenolic substances that bind to proteins, amino acids, alkaloids, and precipitate them and are highly expected antimicrobial biomolecules (Kurhekar, 2016), and previous studies have demonstrated the *in vitro* anti-*H. pylori* activity of the compounds (Wang, 2014; Cardoso et al., 2018). According to Funatogawa et al.'s work, monomeric hydrolyzable tannoids inclusive of tellimagrandin I and II exhibit strong antibacterial activity by damaging the membrane of *H. pylori* (Funatogawa et al., 2004). In addition, tannoids can also serve as inflammatory mediators by reducing nitric oxide levels and exerting anti-inflammatory activity on gastric mucosa (Cardoso et al., 2018).

Curcumin, the principal ingredient isolated from *turmeric*, has been used in many Asian regions as an herbal remedy for various diseases (Kwiecien et al., 2019). *In vivo*, an experiment in a mouse model has proven a notably reduced number of *H. pylori* colonizing mucosa, which may be attributed to decreased activity of lipid peroxide, MPO and urease and increased level of immunomodulation (De et al., 2009). Recent evidence offered by randomized clinical trials also revealed that triple therapy with curcumin can improve symptoms of dyspepsia, attenuate oxidative stress, and prevent mucosa against inflammation, and some showed that adjunctive therapy with curcumin can improve the eradication rate of *H. pylori* (Khonche et al., 2016; Judaki et al., 2017). Additionally, the good interaction between curcumin and targeted virulence factors such as Ure α / β subunits is implicated in the prevention of *H. pylori* survival and colonization (Shetty et al., 2021). However, the oral bioactivity of curcumin as an alternative remedy for *H. pylori* elimination is weakened by its limited aqueous solubility and retention time, for which an effective delivery system is needed to ensure enough therapeutic window at the site of infection, and chitosan (CS) polymer may be a practical solution (Ejaz et al., 2022).

4.2.3 Alkaloids

Alkaloids are a wide range of naturally occurring molecules that contain at least one nitrogen atom and several hydrogen-carbon groups in their structures, forming heterocyclic rings. To date, over 10,000 kinds of alkaloids have been identified among 300 plant families (Mahapatra et al., 2019). A variety of compounds in alkaloid families have shown multiple therapeutic effects, for example, their antitumor, antimicrobial, anti-inflammatory and antidiabetic properties (Wu et al., 2018). Numerous previous studies have noted diverse representatives of pharmaceutically

used alkaloids, among which some are recognized as promising antimicrobial agents, such as berberine, coptisine, palmatine, and quinolone alkaloids (Hamasaki et al., 2000; Wen et al., 2022). Some studies have focused on the mechanisms of anti-*H. pylori* properties, which will be discussed in the following section.

The inhibition of urease activity is a common mechanism of alkaloids against *H. pylori*. According to the significant reduction in anti-urease activity after adding sulfhydryl-containing reagents or competitive Ni²⁺-binding restrainers, sanguinarine (a natural alkaloid enriched in *Zanthoxylum nitidum*) is supposed to suppress urease by targeting Ni²⁺ and thiol (Lu et al., 2022). Similarly, coptisine could also interact with both active center Ni²⁺ and sulfhydryl groups in amino acid residues to inhibit urease activity (He et al., 2022b). In addition to urease inhibition, coptisine also demonstrates various antibacterial mechanisms, including decreasing Cag expression and inducing DNA fragmentation (Tang et al., 2023). Moreover, many alkaloids, such as coptisine and squalamine, can cause disruption of the cell membrane of *H. pylori*, which is especially beneficial for synergistic therapy with commonly used antibiotics (such as amoxicillin), providing extra portals for some compounds to enter the bacteria (Krzyzek et al., 2021; Tang et al., 2023). Piperine, an alkaloid present in black pepper, has shown a diminution of *H. pylori* motility as well as adhesion to gastric cells, contributing to the suppression of *H. pylori* growth (Tharmalingam et al., 2014).

Like other natural products, alkaloids can also exert diverse anti-inflammatory effects, including the reduction of certain proinflammatory cytokines (IL-2, IL-6, IL-17, CXCL1) (Tang et al., 2023), the regulation of macrophage activity (Yang et al., 2021), and the modulation of inflammatory signaling pathways (Alam et al., 2019; Haftcheshmeh et al., 2022). In addition to anti-inflammatory properties, some studies have investigated the gastroprotective effects of alkaloids and reported improvements in the ulcer area of rats, with increased prostaglandin E₂ (PGE₂) as well as decreased platelet-activating factor (PAF) (Wang et al., 2017). All of these features may relieve the symptoms and health hazards induced by *H. pylori*.

5 The Merits of ethnomedicine and further perspectives

Given that only a limited fraction of plant species have been scientifically studied, there still lies great potential in discovering new promising drugs, which are likely to be included in ethnomedicine and are perceived to be an ideal substitute or supplement for current recommended treatment.

Ethnomedicine distinguishes itself from traditional therapies by its accessibility, relatively affordable price and widespread availability. In addition, many consumers perceive herbal medicine as a more natural and thus safer option than synthetic drugs to address diseases, particularly in regions where traditional medicine has been widely practiced for a long time. Although herbal medicine cannot necessarily avoid adverse effects, evidence has proven the safety advantages of natural products over the traditional therapies of combining multiple antibiotics (Deng et al., 2022; Liu et al., 2022). Some studies have investigated the efficacy and safety of the

combination of standard therapy and some ethnomedicine medications, which was demonstrated to have a higher eradication rate and fewer side effects than using an antibiotic-based regimen (Bao et al., 2022). In addition to the eradication efficiency, combination medication possesses the superiority of relieving the symptoms of *H. pylori*-associated gastritis, with the concept of overall adjustment and multitargeting curative effects (Li et al., 2021). Moreover, another appealing reason for adding ethnomedicine to treatments is that pharmaceutical synergism may lessen the likelihood of antibiotic resistance, which can also be attributed to the multiple target effects of ethnomedicine (Ngo et al., 2013; Li et al., 2021). Ultimately, the use of ethnomedicine has noteworthy value in the *H. pylori* eradication of specific populations. For example, it is relatively inappropriate to employ conventional antibiotic-based regimens in vulnerable elderly individuals who would be unable to endure the possible adverse effects of high-dose antibiotics and acid suppression. Thus, ethnomedicine, with milder side effects, is regarded as an ideal therapeutic solution.

Apart from the aforementioned advantages from the patients' perspective, the screening process for new pharmaceuticals also gains from the higher success rate of natural product libraries and the information provided by ethnomedicine. Natural products already possess biological functions since they are mainly primary and secondary metabolites, which leads to a higher possibility of discovering biologically appropriate compounds in natural product libraries than exploring traditional combinatorial chemistry (Sukuru et al., 2009; Buenz et al., 2018). The screening of polyketide metabolites is a good illustration of the efficiency with a hit rate of 0.3% (more than 20 commercial drugs in just over 7000 known structures), while the hit rate for HTS of synthetic compound libraries is less than 0.001% (Weissman and Leadlay, 2005). Additionally, with its empirical knowledge and historical trials, the ethnomedicine system may also facilitate the identification of bioactive compounds by indicating possible screening directions (Buenz et al., 2018).

With the advances of new technologies such as virtual screening, bioinformatics, and artificial intelligence, bioprospecting is currently equipped with innovative powerful tools and undergoing rapid evolution. For instance, in the work of He et al., virtual screening and molecular modeling were utilized to identify ligands that interact with CagA protein to discover potential natural compound candidates (He et al., 2022a). Bioinformatics approaches, including genomics, proteomics, and metabolomics, are also widely implemented in natural product biosynthesis and production, and some researchers bet on artificial intelligence projects for drug innovation inspiration (Ngo et al., 2013; Merwin et al., 2020; Zhang et al., 2020).

While ethnomedicine may provide valuable insights into potential natural remedies or complementary approaches, there are several challenges associated with the development of ethnomedicine as a treatment for *H. pylori*. It has been generally accepted that the premise of TCM treatment is syndrome differentiation, which is the cornerstone of treatment. However, due to the complexity of TCM syndrome types, it is difficult to develop a widely accepted TCM syndrome type differentiation system at present (Li et al., 2021; Elbehiry et al., 2023). Thus, it is

necessary to summarize the experience from current clinical practice and integrate experts' opinions to form consensus about syndrome differentiation for *H. pylori*-associated diseases. In addition, basic animal experimental studies were also limited by the complexity of TCM syndrome types. Currently, most animal experimental studies on disease-syndrome combinations only have a single syndrome type, which is inconsistent with the complex syndrome type of TCM that is more common in clinical practice. Future studies should explore and develop new research models containing complex disease-syndrome combinations to be in line with the current majority clinical practice. Furthermore, the combination of natural products and Western medicine is becoming increasingly common in modern clinical research. This approach can achieve the goal of increasing efficiency and reducing toxicity, highlighting the advantages of combination therapy. Phytochemicals from ethnomedicine formulas and their compositional herb medicines exhibit great potential for the development of novel anti-*H. pylori* drugs. However, the current investigation of the combination of natural products and Western medicine for treating *H. pylori* is still insufficient. Researchers should try to combine some herbal monomers that have clear chemical structures or active ingredients in formulas to develop novel drugs for treating *H. pylori* infection based on the compatibility principle of ethnomedicine prescriptions (Li et al., 2021). Last, the effectiveness and safety of natural products can vary significantly (Pérido et al., 2015), and it is important to consider potential interactions with other medications and any possible side effects. There is currently little clinical trial data regarding their safety and efficacy. Therefore, more multicenter, double-blind, randomized, and controlled clinical trials should be conducted to validate the effectiveness and safety of these natural product prescriptions in the treatment of *H. pylori*. Well-designed clinical trials and experimental studies would be beneficial for facilitating a better understanding of the mechanism of natural products and promoting the modernization of natural products in the treatment of *H. pylori*-associated diseases (Yang et al., 2023).

6 Summary

Confronting the challenge presented by *H. pylori* antibiotic resistance, ethnomedicine is a particularly noteworthy choice of complementary therapy. The scientists and practitioners should continue working collaboratively to bring this ancient wisdom up-to-date and make full use of it in *H. pylori* eradication. In this review, we comprehensively summarized the anti-*H. pylori* function and mechanisms of natural products, and analyzed the therapeutic advantages of incorporating ethnomedicine into anti-*H. pylori* regimens, including safety, accessibility, efficiency and restriction of antibiotic resistance. Natural products can also provide clues for novel antimicrobial drug discovery. This review can provide insights into the development of natural products and expand the therapeutic options available for *H. pylori* eradication. However,

there is still some limitation. The language of the included studies was limited to English, so some potential eligible studies published in other languages might be neglected.

Author contributions

RD: Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. XC: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Software. SZ: Investigation, Writing – review & editing, Methodology, Validation. QZ: Investigation, Project administration, Supervision, Writing – review & editing, Resources. YS: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing, Methodology, Project administration.

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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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Phage P2-71 against multi-drug resistant *Proteus mirabilis*: isolation, characterization, and non-antibiotic antimicrobial potential

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Proteus mirabilis, a prevalent urinary tract pathogen and formidable biofilm producer, especially in Catheter-Associated Urinary Tract Infection, has seen a worrying rise in multidrug-resistant (MDR) strains. This upsurge calls for innovative approaches in infection control, beyond traditional antibiotics. Our research introduces bacteriophage (phage) therapy as a novel non-antibiotic strategy to combat these drug-resistant infections. We isolated P2-71, a lytic phage derived from canine feces, demonstrating potent activity against MDR *P. mirabilis* strains. P2-71 showcases a notably brief 10-minute latent period and a significant burst size of 228 particles per infected bacterium, ensuring rapid bacterial clearance. The phage maintains stability over a broad temperature range of 30–50°C and within a pH spectrum of 4–11, highlighting its resilience in various environmental conditions. Our host range assessment solidifies its potential against diverse MDR *P. mirabilis* strains. Through killing curve analysis, P2-71's effectiveness was validated at various MOI levels against *P. mirabilis* 37, highlighting its versatility. We extended our research to examine P2-71's stability and bactericidal kinetics in artificial urine, affirming its potential for clinical application. A detailed genomic analysis reveals P2-71's complex genetic makeup, including genes essential for morphogenesis, lysis, and DNA modification, which are crucial for its therapeutic action. This study not only furthers the understanding of phage therapy as a promising non-antibiotic antimicrobial but also underscores its critical role in combating emerging MDR infections in both veterinary and public health contexts.

KEYWORDS

bacteriophage therapy, multidrug-resistant *proteus mirabilis*, non-antibiotic antimicrobial strategies, phage genomics and host interactions, phage-bacteria interaction

1 Introduction

P. mirabilis, a Gram-negative bacterium, is renowned for its remarkable swarming motility on solid surfaces. This bacterium is a frequent urinary tract pathogen, particularly among patients with prolonged catheter usage (O'Hara et al., 2000; Schaffer and Pearson, 2015). Besides urinary tract infections (UTIs), *P. mirabilis* also causes other infections including superficial skin and respiratory tract infections (Jacobsen and Shirliff, 2011). The escalating emergence of MDR strains of *P. mirabilis* underscores the pressing need to discover new and effective approaches for combating microbial infections (Algammal et al., 2021; Kang et al., 2021; Li et al., 2022). The emergence of MDR *P. mirabilis* strains in dogs presents a significant public health concern, particularly due to the close relationship between dogs and humans (Decôme et al., 2020; El-Tarabili et al., 2022).

The presence of MDR bacteria in UTIs reduces the efficacy of antibiotic treatments (Flores-Mireles et al., 2015). Phages have emerged as potentially promising alternatives for managing antibiotic-resistant UTIs. Recent research has shown that phages can effectively target bacteria in UTIs and disrupt their biofilms (Melo et al., 2016; Yazdi et al., 2018). Scientists suggest using phage therapy as a strategy to prevent and treat UTIs caused by MDR bacteria. However, phage-resistant strains continue to emerge despite the use of phage cocktails and phage-antibiotic combinations (Chegini et al., 2021; Oyejobi et al., 2022). To effectively address this problem, further foundational studies are essential to explore the interplay between phages and their hosts and the potential of phages in managing UTIs. Additionally, understanding the stability and activity of phages within the challenging environment of the urinary tract, especially in artificial urine which simulates the conditions of the urinary system, is vital for the development of effective phage therapy.

In this study, we isolated a lytic *P. mirabilis* phage named P2-71 from dog feces. We conducted a comprehensive evaluation that included transmission electron microscopy for morphology assessment, host range determination, one-step growth curve analysis, temperature and pH stability assessments. Additionally, we performed killing curve analyses in both LB broth and artificial urine to characterize its bactericidal properties. A detailed genomic analysis of P2-71 was also undertaken. Our extensive research into P2-71 highlights its potential as an effective non-antibiotic antimicrobial agent, demonstrating its relevance in addressing the pressing global issue of drug-resistant bacterial infections.

2 Materials and methods

2.1 Experimental bacterial strains

This investigation employed MDR *P. mirabilis* strains retrieved from dog fecal matter, which are maintained within our lab's repository. *P. mirabilis* 37, serving as the host strain for phage P2-71, was among these strains. The multi-drug resistance (MDR) status of these *P. mirabilis* strains was determined based on resistance to at least three different classes of antimicrobial agents,

as per the standards set by the Clinical and Laboratory Standards Institute (CLSI). Details regarding the bacterial strains, their origins, the accession numbers correlating to the 16S rRNA gene sequences, and the specific resistance profiles can be found in (Liu et al., 2023). A brief summary of the strains used is provided in (Supplementary Table 1). Additionally, four standard strains - *Salmonella enterica* H9812, *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* ATCC, 25923, and enterotoxigenic *Escherichia coli* (ETEC) ATCC, 25922 - were procured from the American Type Culture Collection (ATCC).

To initiate bacterial growth, we cultured the strains in Lysogeny Broth (LB) and then maintained them in an incubator at a stable 37°C with agitation at 160 rpm throughout the night to promote favorable growth conditions.

2.2 Isolation and purification of phage P2-71

The phage P2-71, retrieved from dog fecal matter from the Protect Beastie Facility in Sichuan, underwent an extraction process starting with the blending of 1 gram of fecal content with 1 milliliter of SM buffer. This blend was then subjected to centrifugation at 8000×g for a duration of 5 minutes at a temperature of 4°C, and the supernatant obtained was passed through a filter with 0.22 µm pores to discard solid particles. This clarified supernatant was then combined with a logarithmic-phase *P. mirabilis* 37 culture and maintained for 6 hours at a temperature of 37°C with continuous shaking at 220 revolutions per minute. Post-incubation, a centrifuge run at 12000×g for 3 minutes at 4°C was performed to separate the supernatant. This supernatant was then diluted in a gradient, mixed equally with a bacterial culture in the logarithmic-phase, and set aside for a quarter of an hour. Afterward, the diluted mixture was incorporated into 4 milliliters of a semi-solid LB substrate employing the double agar layer technique, before being applied to firm LB agar bases. These bases were then kept in an incubator set at a stable 37°C for an overnight period. Distinct plaques, which signify phage activity, were meticulously extracted into SM buffer and amalgamated thoroughly. To guarantee a pure phage specimen, this meticulous extraction was conducted multiple times.

The purification and storage of phage P2-71 were modified based on the method described by Mondal et al (Mondal et al., 2023), starting with the mixture of the *P. mirabilis* 37 logarithmic-phase culture with phage P2-71 at an MOI of 0.1, followed by the addition of 2 ml of LB medium. This mixture was then incubated overnight at 37°C with constant shaking at 220 rpm. After this period, a centrifugation at 8000×g for 10 minutes at 4°C was performed, and the supernatant was kept. The sample was then subjected to an ultracentrifugation at 30000 rpm for 4 hours at 4°C. Subsequently, the pellet was resuspended and subjected to sucrose density gradient centrifugation under identical conditions for 4 hours, which facilitated the collection of the phage band. This collected phage layer was then diluted twofold and underwent a low-speed centrifugation at 8000×g for 10 minutes at 4°C to extract excess sucrose. This step of sucrose removal was repeated three times. The final concentrated phage P2-71 was stored at a refrigeration temperature of 4°C.

2.3 Host range

To assess the host range of phage P2-71, logarithmic-phase bacterial cultures and a freshly prepared phage solution were used. Serial dilutions (gradient dilutions) of the phage were prepared, and these were mixed with the bacterial cultures in a 1:1 ratio, allowing the mixtures to stand for 15 minutes to facilitate interaction. Subsequently, each mixture was combined with 4 mL of semi-solid LB medium and carefully overlaid onto solid LB medium plates. These plates were then placed in a constant-temperature incubator at 37°C and incubated overnight. The presence of clear zones or plaques on the plates indicated bacterial susceptibility to lysis by phage P2-71. Bacterial strains that displayed such clear plaques were classified as susceptible to the phage, evidencing the lytic capability of phage P2-71 against these bacterial hosts.

2.4 Biological characteristics of phage P2-71

Investigation into the structural composition of phage P2-71 was carried out through a transmission electron microscope (TEM) 2100plus (JEOL, Japan), adhering to sample preparation guidelines outlined in established research (Hao et al., 2023).

A one-step growth curve was utilized to determine the latent period, rise phase, plateau period, and burst size of phage P2-71. Newly reactivated phage particles at a multiplicity of infection (MOI) of 0.01 were amalgamated with an actively dividing *P. mirabilis* 37 culture (2×10^8 CFU/mL), followed by a resting period of 15 minutes to facilitate phage-bacteria engagement. Post-rest, the blend was centrifuged at 13,000×g for a brief span of 2 minutes to segregate non-adsorbed phages. The supernatant was then removed, and the resultant pellet was reintroduced into LB broth and incubated at 37°C with a shaker set at 220 rpm, marking the commencement of the observation as zero time. Sampling was done initially at 5-minute intervals for the first 20 minutes, followed by 10-minute intervals, with immediate gradient dilution for phage titer analysis. The burst size was determined by the quotient of the released phage count to the initial infected bacterial cell count during the latency phase (D'Andrea et al., 2017).

The pH stability of phage P2-71 was ascertained through a battery of pH conditions ranging from 1.0 to 14.0 in SM buffer. Each buffer, containing the phage at a starting titer of 10^9 PFU/mL, was incubated at a stable 37°C for an hour in a temperature-controlled water bath. Subsequent titer evaluations were performed employing the double layer agar plate technique.

The thermal endurance of the phage was gauged by exposing phage suspensions at a concentration of 10^9 PFU/mL to varied constant heat settings (30, 40, 50, 60, 70, and 80°C) for periods ranging from 30 to 60 minutes. Determination of phage concentrations post-heat exposure was standardized to the 30°C, 60-minute control. To ensure the validity of the results, each assessment was replicated threefold.

2.5 Time-kill assay

The *in vitro* bactericidal activity of phage P2-71 was evaluated using a time-kill assay, adapted from the method described by (Ciacci et al., 2018, 101). The phage titer was adjusted to create a series of MOI levels including 10, 1, 0.1, and 0.01. For each MOI, 100 µl of phage solution was mixed with 100 µl of *P. mirabilis* 37 in the logarithmic growth phase (2×10^8 CFU/mL). The resulting mixtures were then transferred into 10 ml of LB liquid culture medium. These cultures were incubated at 37°C with constant agitation at 220 rpm. The optical density at 600 nm (OD600) was measured at 10-minute intervals using a Thermo Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, United States). This procedure was performed in triplicate to ensure the reproducibility of the results.

2.6 Stability of phage P2-71 in artificial urine

The stability of phage P2-71 was evaluated in an artificial urine solution (Phygene, Fuzhou, China) with a pH of 5.7, to closely replicate the urinary tract environment. After the inoculation of the phage, the suspension was maintained at a steady temperature of 37°C for incubation. To assess the stability of P2-71, samples were collected at specified time intervals: 0, 1, 2, 4, 6, 12, 18, and 24 hours post-inoculation. At each time point, the titer of phage P2-71 was quantified using the double-layer agar plate method. This assay was designed to understand the persistence and stability of P2-71 in a simulated urinary environment, providing insights into its potential for clinical applications.

2.7 Time-kill assay of P2-71 in artificial urine

Following the protocol outlined in Section 2.5, the bactericidal effect of phage P2-71 on *P. mirabilis* 37 was further examined in an artificial urine environment, aiming to simulate conditions similar to the urinary tract. Cultures of *P. mirabilis* 37 in their logarithmic growth phase were first centrifuged at 5000×g for 20 minutes at 4°C. After discarding the supernatant, the bacterial pellet was resuspended in PBS. This centrifugation and resuspension process was repeated once more to ensure thorough removal of the LB medium. The bacterial concentration was then adjusted to 2×10^8 CFU/mL, readying it for combination with phage P2-71 at MOI levels of 10, 1, 0.1, and 0.01.

These mixtures were subsequently transferred into 10 ml of artificial urine and incubated in a temperature-controlled shaker at 37°C, with continuous agitation at 220 rpm. The interaction between the phage and bacteria was monitored for a period of 180 minutes, and OD600 was measured at 10-minute intervals. Each set of conditions was replicated in triplicate to ensure the consistency and reproducibility of the experimental data.

2.8 Genomic analysis

The genomic sequencing of phage P2-71 was executed utilizing the PE150 module on the NovaSeq, 6000 apparatus. Subsequent bioinformatics analysis was undertaken, initiating with Fastp (Chen et al., 2018) for the primary data processing which encompassed adapter trimming, discarding low-quality sequences, and filtering out high N ratio reads. The *de novo* assembly was conducted via metaSPAdes (Nurk et al., 2017), with a series of k-mer lengths tested to achieve optimal results. Alignment of clean reads to the assembled genome for coverage assessment was done using bwa (Li and Durbin, 2009).

Annotation of open reading frames (ORFs) was conducted using Rapid Annotation using Subsystem Technology (RAST) (Brettin et al., 2015) and Prokka (Seemann, 2014), complemented with blastp for Supplementary Annotations. The Virulence Factor Database (VFDB) (Liu et al., 2022) was utilized for virulence factors identification, while predictions for potential drug-resistance genes were made through the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2023). tRNA genes were forecasted using tRNAscan-SE 2.0 (Chan et al., 2021), and CRISPR arrays along with Cas proteins were identified via CRISPRCasFinder (Couvin et al., 2018). Visualization of the phage P2-71 genomic map was facilitated by Proksee (Grant et al., 2023).

Phylogenetic investigations were conducted with MEGA11 software (Tamura et al., 2021, 11), particularly focusing on the terminase large subunit gene of phage P2-71 and its closely related sequences. The Neighbor-Joining method (Saitou and Nei, 1987), was employed to construct the phylogenetic tree, and the tree's robustness was verified through, 1000 bootstrap tests (Felsenstein, 1985). The calculation of evolutionary distances was executed using the Maximum Composite Likelihood approach (Tamura et al., 2004). This analysis included 16 sequences, spanning, 2012 positions after removing all ambiguous bases, with pairwise

deletion as the chosen strategy. Comparative genomic insights were graphically portrayed using Easyfig 2.2.5 (Sullivan et al., 2011). Accession numbers for the phage genomes included in this phylogenetic analysis are available in (Supplementary Table 2).

3 Result

3.1 The characteristics of phage P2-71

In the current investigation, phage P2-71 was successfully isolated from *P. mirabilis* 37. The isolation technique employed the Double-Layer Agar method, resulting in the formation of distinct, small circular, and translucent plaques (Figure 1A). Transmission electron microscopy (TEM) provided detailed insights into the morphology of phage P2-71, illustrating a dodecahedral head with an average diameter of 62 ± 3.0 nm and a tail measuring an average length of 234 ± 10 nm (Figure 1B), characteristic of the Siphoviridae family.

3.2 Assessment of biological characteristics of phage P2-71

The one-step growth curve of phage P2-71 was characterized by a 10-minute latency phase, succeeded by a 20-minute exponential rise phase, culminating in a plateau phase that was sustained for approximately 70 minutes. The estimated burst size was determined to be 228 phage particles per infected bacterium (Figure 2A).

Thermal stability assays revealed that phage P2-71 maintained stable titers between 30°C and 50°C. A discernible decrease in activity was observed at 60°C, indicating a threshold for thermal stability (Figure 2B).

Furthermore, the phage's resilience across a broad pH spectrum was evaluated. Phage P2-71 displayed considerable stability within a

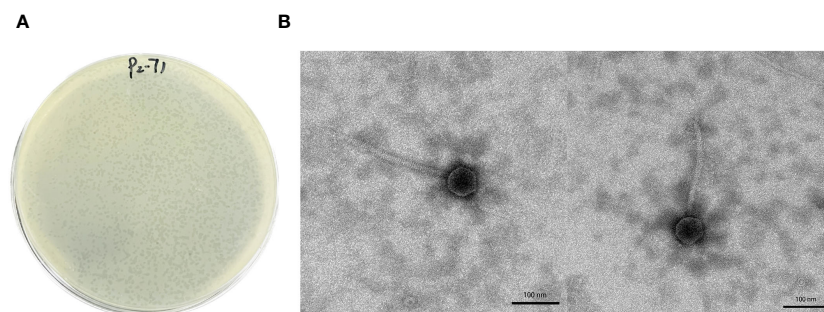


FIGURE 1

Morphological analysis of phage P2-71. (A) Plaque Morphology of Phage P2-71: This panel shows a Petri dish following the Double-Layer Agar technique used for phage isolation, displaying the characteristic small, circular, and translucent plaques formed by phage P2-71 against a lawn of *P. mirabilis* 37. These plaques are indicative of lytic activity where the phage has infected and cleared areas of bacterial growth on LB agar medium. (B) Presented here are high-resolution TEM images of phage P2-71, which unveil its structural morphology. The phage is observed to have a dodecahedral head, approximately 62 ± 3.0 nm in diameter, attached to a lengthy tail measuring about 234 ± 10 nm. These dimensions and shape are typical features of phages belonging to the Siphoviridae family, suggesting its classification within this group. Scale bars represent 100 nm, providing a reference for size estimation.

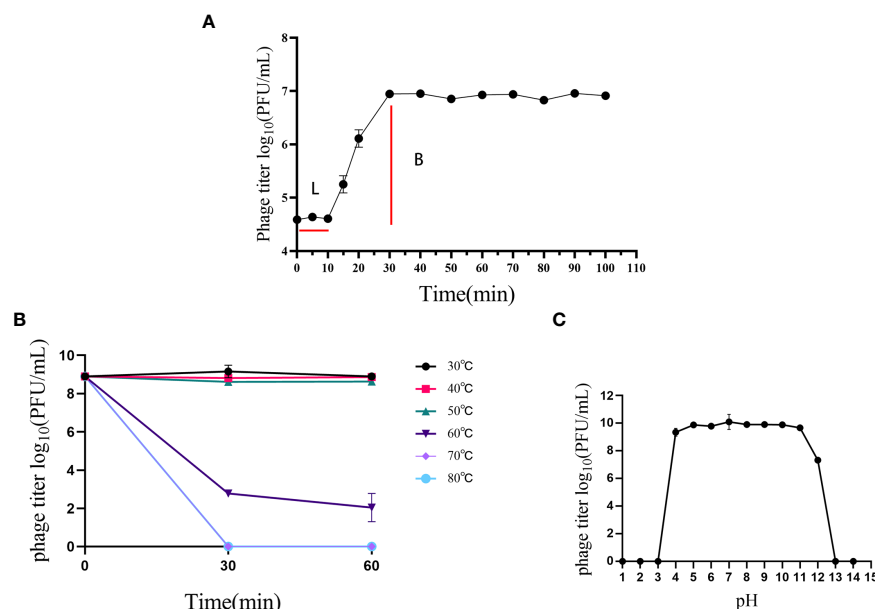


FIGURE 2

Biological characterization of phage P2-71. **(A)** Growth Kinetics: The one-step growth curve of phage P2-71 showing an initial latency phase of 10 minutes followed by a rapid rise phase over the next 20 minutes, leading to a plateau that persists for about 70 minutes, indicative of the phage's replication cycle within the host bacteria. **(B)** Thermal Stability: Phage P2-71 demonstrates consistent phage titers indicating stability from 30°C to 50°C. At 60°C, a marked decline in phage titer suggests a limit to thermal tolerance, beyond which phage activity is compromised. **(C)** pH Stability: Stability testing across a pH gradient shows that phage P2-71 maintains stability within a pH range of 4 to 11, with a slight reduction in activity observed at pH 12, demonstrating the phage's adaptability to various pH conditions. Error bars represent the standard deviation (SD) from three independent experiments.

pH range of 4 to 11, with marginal activity persisting at pH 12. This robustness to pH variations underscores the potential for versatile applications of phage P2-71 (Figure 2C).

3.3 Killing assay

Logarithmic phase cultures of *P. mirabilis* 37 were treated with phage P2-71 at MOIs of 10, 1, 0.1, and 0.01. Optical density at 600 nm (OD600) was periodically measured using a spectral scanning multimode reader to monitor bacterial growth. The resulting killing curves demonstrated a progressive inhibition of bacterial proliferation post-phage addition, with the extent of reduction being directly proportional to the respective MOI. Remarkable bacterial suppression was observed consistently, even at the lowest tested MOI of 0.01 (Figure 3).

3.4 Host range

Different MDR strains of *P. mirabilis* and four standard strains of bacteria (*Salmonella enterica* H9812, *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* ATCC, 25923, and enterotoxigenic *Escherichia coli* ATCC, 25922 (ETEC) were assessed using double layer agar plate method. The results revealed that phage P2-71 has lysis ability against a portion of MDR strains of *P. mirabilis* (9/36)

but does not exhibit the lysis ability to the four Standard strains of bacteria mentioned above (Supplementary Table 3).

3.5 Stability of phage P2-71 in artificial urine

The stability assessment of phage P2-71 over a 24-hour period in artificial urine demonstrated no significant change in phage concentration. The titer remained stable across all measured time points, including 1, 2, 4, 6, 12, 18, and 24 hours post-inoculation. Statistical analysis using one-way ANOVA confirmed that the observed variations in the data were within the standard deviation range, and the differences between groups did not reach statistical significance, with p-values greater than 0.05 (Figure 4).

3.6 Time-kill assay of P2-71 in artificial urine

The application of phage P2-71 to *P. mirabilis* 37 cultures in artificial urine exhibited a MOI-dependent inhibition of bacterial growth. The control group showed a slight increase in OD600. In contrast, phage-treated groups demonstrated an immediate decline in OD600 after phage addition, with a more pronounced effect at the highest MOI. Following the initial reduction, OD600 values

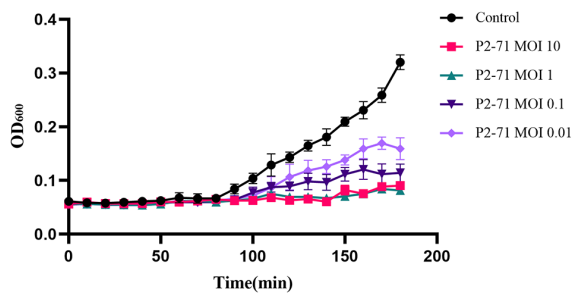


FIGURE 3

Killing assay of phage P2-71 to lyse *P. mirabilis* 37. This figure presents the effect of phage P2-71 on *P. mirabilis* 37 growth at varying multiplicities of infection (MOIs) of 10, 1, 0.1, and 0.01. The control group (black line) consists of bacterial culture without phage treatment. Each line represents the change in optical density at 600 nm (OD₆₀₀) over time, indicating bacterial growth. A noticeable reduction in bacterial proliferation is evident in cultures treated with phage P2-71, with the degree of inhibition correlating to the MOI used. The lowest MOI of 0.01 still exhibits noticeable suppression of bacterial growth. Data points are the mean of triplicate measurements, with error bars indicating SD. These results illustrate the potent bactericidal activity of phage P2-71 across a range of MOIs.

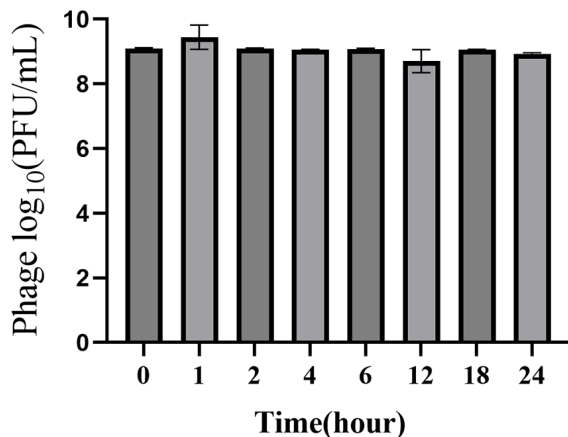


FIGURE 4

Stability of phage P2-71 over a 24-hour period in artificial urine. The bar chart illustrates the titer of bacteriophage P2-71 at various time points following inoculation into artificial urine. Samples were collected immediately after inoculation (0 hours) and subsequently at 1, 2, 4, 6, 12, 18, and 24 hours post-inoculation. Error bars represent the SD from three independent replicates. Statistical analysis using one-way ANOVA revealed that variations in phage titers over the 24-hour period were not statistically significant, indicating consistent stability of phage P2-71 in artificial urine.

across phage-treated samples plateaued, suggesting an equilibrium between phage action and bacterial growth. Lower MOI treatments resulted in a less marked decrease in OD₆₀₀ (Figure 5).

3.7 Genomic insights and comparative analysis of phage P2-71

The genomic analysis of phage P2-71 has revealed a genome comprising 58,706 base pairs (bp) with a G+C content of 46.87%.

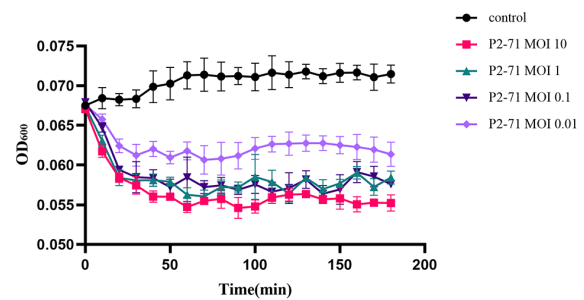


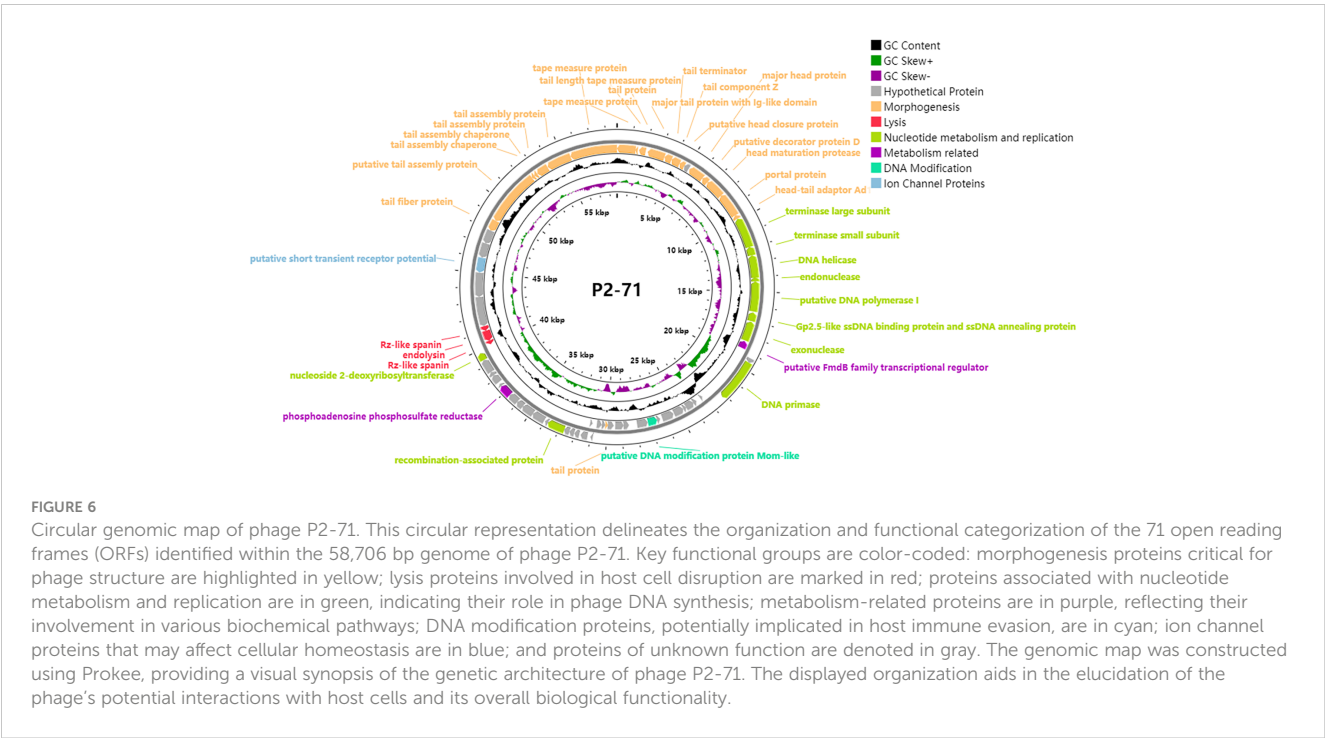
FIGURE 5

Killing assay of phage P2-71 to lyse *P. mirabilis* 37 in artificial urine. This graph displays the OD₆₀₀ measured over 180 minutes following inoculation. The control group (black) shows a slight uptrend in OD₆₀₀, indicative of minimal bacterial growth. In contrast, the phage-treated groups experience an immediate decline in OD₆₀₀, with the highest MOI of 10 (red) showing a marked reduction in bacterial growth. Following this initial drop, the OD₆₀₀ values for the phage-treated samples plateau, indicating a state of equilibrium between phage activity and bacterial growth. Error bars denote SD from three replicates.

Analysis of this genome has identified 71 open-reading frames (ORFs) (Supplementary Table 4), each classified according to their predicted functions derived from sequence analysis, enhancing our understanding of the phage's genetic blueprint. These ORFs span a diverse array of roles, from contributing to the phage's structural integrity to mediating critical enzymatic functions within its life cycle. Notably, ORF1 is characterized as encoding a protein that correlates with the dimensional attributes of the phage tail, an attribute likely significant in host cell recognition. ORF4 and ORF65, implicated in tail morphology, suggest a contributory role to the phage's affinity for specific host interactions. The identification of ORF57 and ORF58 as potential encoders of lysis-associated proteins posits their involvement in the liberation of nascent phage particles following host cell infection. Additionally, the characterization of ORF31, bearing similarity to DNA-modifying enzymes, implies a possible function in the phage's interaction with host genomic material, which may influence its replication efficacy and host immune evasion strategies. The complete genomic sequence of phage P2-71 has been deposited in the NCBI database, with the accession number OR672055.1. The genomic map (Figure 6) categorizes the identified proteins by their predicted functions and provides a visual overview of the phage P2-71 genome.

Our phylogenetic analysis, based on the terminase large subunit gene sequences, positions phage P2-71 within a distinct clade, denoting its close genetic relatedness with particular *Proteus* phages. The constructed evolutionary tree demonstrates P2-71 clustering closely with *Proteus* phage Isf-Pm1 (Mirzaei et al., 2022). This clustering is supported by robust bootstrap values, which suggest a high level of confidence in this particular branch of the tree. The observed phylogenetic branching patterns and genetic distance metrics provide insights into P2-71's evolutionary lineage and its relationships with other closely related phages (Figure 7).

Genomic comparison studies facilitated by Easyfig 2.2.5 have revealed key aspects of phage P2-71's genetic structure. Synteny



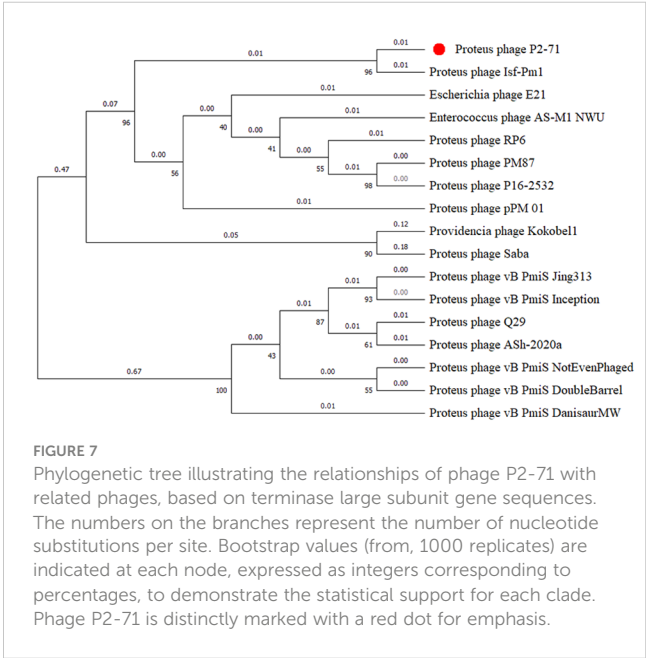
analysis between P2-71 and the closely related *Proteus* phage Isf-Pm1 demonstrates a high degree of genomic conservation, with extensive synteny blocks indicative of recent evolutionary divergence. In contrast, the comparison with the more distantly related phage Q29 shows significant genomic rearrangement and a marked reduction in conserved sequences, suggesting an evolutionary pathway with considerable divergence. These comparative genomic insights reveal the variability among phages and underscore distinctive genomic attributes of P2-71 that may influence its phage life cycle and host specificity (Figure 8).

4 Discussion

The rise of MDR bacterial strains presents a substantial risk to public health, particularly in the case of UTIs caused by MDR bacteria in elderly patients, which add a substantial burden to care (Garcia-Bustos et al., 2021). In the veterinary field, *P. mirabilis*, a pathogen associated with UTIs in companion animals, has been identified as a cause of health issues in these animals (Jacobsen et al., 2008). Significantly, there is a notable clonal relatedness in urinary pathogenic *P. mirabilis* observed across humans and their domestic pets, suggesting the potential for cross-species transfer of UTI-inducing *P. mirabilis* (Marques et al., 2019). MDR *P. mirabilis* strains have been identified in diverse animal species, including ducks (Algammal et al., 2021), cattle (Jayol et al., 2018), and chickens (Li et al., 2022). In this study, both the MDR *P. mirabilis* strain and phage p2-71 were isolated from a stray dog center. Given the close contact between stray dogs and humans, it is reasonable to speculate that MDR *P. mirabilis* could potentially be transmitted from stray dogs to humans, representing a possible risk to human health. Although our study did not detect whether individuals who came into contact with stray dogs were carriers of *P. mirabilis*, the potential risk of transmission from

animals to humans cannot be overlooked. Stray dogs, as animals in close contact with humans, may act as vectors for MDR bacteria, especially in the absence of adequate hygiene measures and surveillance. This finding is particularly relevant in the field of public health, as it underscores the need for monitoring and controlling the transmission of MDR bacteria between animals and humans.

The discovery of antibiotics was a significant breakthrough in medical history, saving countless lives. However, the rise and dissemination of strains resistant to antibiotics have severely challenged the efficacy of antibiotics (Chinemerem Nwobodo et al., 2022). Resistant bacteria proliferate not only in hospital



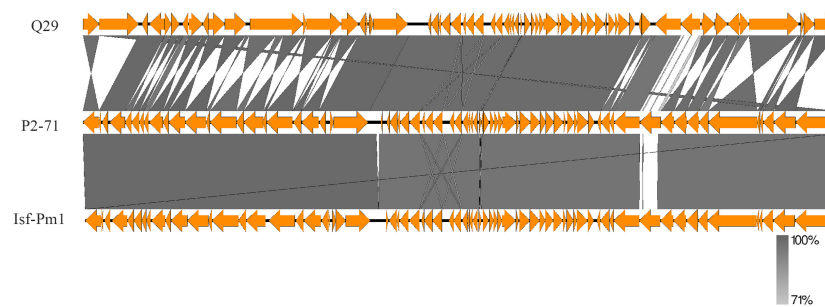


FIGURE 8

Genomic comparison of phage P2-71 with phage Q29 and phage Isf-Pm1 using Easyfig 2.2.5. The synteny plot in the upper panel showcases the genomic relationship between P2-71 and the more distantly related Q29, illustrating significant genomic rearrangement. The lower panel depicts the synteny between P2-71 and Isf-Pm1, highlighting areas of high sequence conservation. The regions of sequence identity are shaded in gray, and gene annotations are represented by arrows, which denote the direction of transcription.

settings but are also widespread in aquatic and agricultural environments (Ondon et al., 2021; Papajová et al., 2022). These bacteria disseminate their resistance genes via Horizontal Gene Transfer (HGT), exacerbating the global issue of antibiotic resistance (Sun et al., 2019). Consequently, the search for alternative strategies to antibiotics has become both urgent and necessary. Phage therapy, as an emerging antimicrobial strategy, has shown immense potential. Phages are highly specific, targeting only particular bacterial hosts, thereby minimizing disruption to the beneficial microbiota in the human body (Lorenzo-Rebenaque et al., 2023), and reducing the development of antibiotic resistance. In this study, we isolated phage P2-71 from the feces of stray dogs, which targets the canine-sourced MDR *P. mirabilis*. This discovery not only provides a new perspective for treating MDR bacterial infections but also lays the groundwork for further research into the application of phages in public health.

Phages infect and lyse their host bacteria through the specific recognition of receptors on the cell wall (Dunne et al., 2021). *P. mirabilis* phage Q29 has demonstrated the capability to lyse 12 out of 27 MDR *P. mirabilis* strains (Hao et al., 2023). In this study, to ascertain the host spectrum of phage P2-71, the double-layer agar technique was utilized, which was able to lyse 9 out of 36 MDR *P. mirabilis* strains preserved in our laboratory. This finding highlights its potential for application. However, it is noteworthy that our host range analysis was limited to a relatively small number of canine-sourced MDR *P. mirabilis* strains available in our laboratory collection. Therefore, the sample size was limited, and it is generally understood that a broader host range often implies a wider scope of potential applications.

The genome size of phage P2-71 is approximately 58,706 bp, containing about 71 predicted ORFs, with a GC content of 46.87%. Phage P2-71 consists of a dodecahedral head approximately 62 ± 3.0 nm in diameter, coupled with a tail measuring about 234 ± 10 nm in length. According to the ICTV classification guidelines, P2-71 belongs to the Siphoviridae family. The tape measure protein (TMP), encoded by ORF1, is a pivotal factor in determining the length of the phage tail. This length is crucial for bridging the distance between the phage and the host cell surface, thereby influencing the phage's ability to penetrate bacterial cell envelopes and reach specific receptors. Studies on the T5

tail tube structure of Siphoviridae phages have revealed the multifunctional nature of TMP, indicating its involvement in host recognition, cell wall perforation, and viral DNA transfer to the host cytoplasm (Arnaud et al., 2017). Furthermore, the evolution of TMPs, characterized by variations in tail length due to gene duplications and losses, underscores the diversity in phage tail architectures and strategies for cell wall recognition and perforation across different phage families (Belcaid et al., 2011). This diversity potentially dictates the range of bacterial cells a phage can infect, with tail length variations influencing the phage's infective capabilities. The presence of ORF4 and ORF65, which encode for the major tail protein with an Ig-like domain and tail fiber proteins respectively, indicates specialized mechanisms for host cell receptor recognition and binding. The Ig-like domain in the major tail protein may play a crucial role in mediating specific interactions with bacterial cell surface proteins, thereby determining the host range of P2-71 (Fraser et al., 2007; Chatterjee et al., 2021). Similarly, tail fiber proteins are instrumental in recognizing and binding to specific receptors on the bacterial surface, a vital process for successful phage attachment and subsequent infection (Nobrega et al., 2018; Farquharson et al., 2021; Taslem Mourosi et al., 2022). ORF57 and ORF58 encode key lysis proteins crucial in the final stage of the phage P2-71 infection cycle, facilitating the release of new virions. ORF58 encodes an endolysin, a phage-encoded peptidoglycan hydrolase, which first degrades the peptidoglycan layer of the bacterial cell wall from within, critical for efficient lysis in G- bacteria (Schmelcher et al., 2012; Rahman et al., 2021). Following this degradation, the Rz-like spanin encoded by ORF57 disrupts the outer membrane, a distinctive feature of G- bacterial cells, allowing the release of progeny phages (Krupović et al., 2008; Kongari et al., 2018). The sequential action of endolysin and Rz-like spanin is essential for effectively breaching the host cell wall and facilitating the release and spread of progeny phages. Understanding the roles of ORF57 and ORF58 in this process provides valuable insights into how P2-71 adapts its infection mechanism for G- bacterial hosts, highlighting its host specificity and infection efficiency. The remaining 37 ORFs are classified as hypothetical proteins, indicating that their functions are still unclear. The unknown functions of these hypothetical proteins provide a wide scope for future research, which may reveal new mechanisms of phage-host interactions.

Phages utilized in therapeutic applications should ideally be devoid of virulence factors and antibiotic resistance genes. Such phages are more favorable for clinical use, as they minimize potential risks to the host and do not facilitate the emergence of drug resistance. In our study, phage P2-71 demonstrated an absence of detectable antibiotic resistance and virulence genes, positioning it as a viable and promising candidate for therapeutic deployment. This characteristic is particularly pertinent in the face of the escalating crisis of antibiotic resistance, underscoring the importance of phages free from resistance genes as they do not contribute to the aggravation of this global health challenge.

In our study, a phylogenetic analysis of the terminase large subunit enzyme revealed that phage P2-71 shares a close genetic relationship with specific *Proteus* phages, particularly Isf-Pm1 (Mirzaei et al., 2022). This connection is supported by high bootstrap confidence values, indicating the robustness of this phylogenetic branch. The high degree of genomic conservation between P2-71 and Isf-Pm1 suggests a recent divergence event from a common ancestor. Such shared characteristics might be a consequence of gene transfer events occurring between different hosts, significantly impacting the adaptability and evolutionary trajectory of these phages. This conservation may elucidate common biological traits shared between the two, such as host range, infection mechanisms, and environmental adaptability. However, the growth curve of Isf-Pm1, with a latency phase lasting 60 minutes and a burst yield of 46 phages per bacterium, differ markedly from those of P2-71, which demonstrates an unusually brief latent period of merely 10 minutes and a notably high burst size, producing 228 phage particles for each infected cell. In terms of growth curves, significant differences were observed between the two phages. Regrettably, the stability and other biological characteristics of Isf-Pm1 have not yet been documented in the literature. Furthermore, a genomic comparison of P2-71 with another phage, Q29 (Hao et al., 2023), revealed substantial rearrangements and divergences, hinting at different evolutionary strategies adopted by phages to adapt to host defense mechanisms and environmental pressures (Dion et al., 2020; Zhang et al., 2023). This genomic plasticity may indicate phages' ability to adapt to variable environments. The marked differences from Q29 might also reflect unique mechanisms employed by P2-71 in its interactions with hosts. The genomic analysis of P2-71 reveals characteristics that might suggest a broader host range potential. However, this must be interpreted in the context of our empirical data, which showed that P2-71 lysed 25% (9 out of 36) of the tested *P. mirabilis* isolates. While these genomic insights hint at wider applications in phage therapy, particularly amid escalating antibiotic resistance, they also underline the necessity for more exhaustive research to fully understand P2-71's infection strategies and precise host range. Our study's exploration into the genetic diversity and evolutionary dynamics of P2-71 provides vital insights, laying a foundation for future research aimed at deciphering how these factors influence the efficacy and specificity of phage therapy against various bacterial strains.

Phage P2-71 demonstrated exceptional thermal stability, maintaining stability across a temperature range of 30°C to 50°C and retaining partial activity even at temperatures as high as 60°C. Such thermal stability is particularly crucial in phage therapy applications,

especially considering the potential impact of temperature fluctuations within the human body on phage efficacy. In contrast, phage Q29 (Hao et al., 2023) maintained partial activity at 65°C, while vB_PmIS-TH (Yazdi et al., 2018) exhibited certain activity even at 75°C, indicating a broader range of temperature adaptability. Regarding pH stability, P2-71 remained relatively stable from pH 4 to 11 and showed some activity even at pH 12. This wide pH stability implies that P2-71 can effectively operate under diverse environmental conditions, which is vital for therapeutic applications due to the significant pH variations in different parts of the human body. By comparison, Q29 maintained a high titer within the pH range of 4 to 11, whereas vB_PmIS-TH sustained activity from pH 3 to 11 but exhibited a significant decline in activity after 24 hours. The latent period of P2-71 is only 10 minutes, with its proliferation rate rapidly escalating within 20 minutes and then maintaining a stable growth phase for approximately 70 minutes. Its burst size is 228 phage particles released per infected cell. This rapid replication capability and substantial burst size mean that P2-71 can quickly proliferate and release a large number of new phages upon infecting host cells, crucial for the swift eradication of pathogens. In comparison, the burst sizes for Isf-Pm1 and Isf-Pm (Mirzaei et al., 2022) are 46 and 666 particles, respectively, while vB_PmIS-TH shows a burst size of 260 PFU per infected cell, similar to P2-71, demonstrating the variability in replication capacities of different phages within host cells. Overall, the biological characteristics of phage P2-71, including its excellent thermal stability, wide pH stability, and large burst size, make it a highly promising candidate for therapeutic applications.

The observed stability of phage P2-71 in artificial urine over 24 hours underscores its potential for phage therapy applications in the urinary tract. This stability, without significant titer fluctuations, aligns with Pereira et al (Pereira et al., 2016), who found phages maintain their concentration in urine, even increasing in the presence of host bacteria, though with diminished inactivation efficacy compared to PBS. In LB medium, the phage P2-71 showcases a potent inhibitory effect on bacterial growth that is maintained throughout the duration of the experiment. The highest MOI of 10 results in a significant and sustained reduction in OD600, indicating a strong and persistent bactericidal activity. This effect is consistent across the various MOIs tested, with even the lowest MOI of 0.01 showing a considerable suppression of bacterial proliferation. Conversely, in the artificial urine environment, the initial impact of the phage is more abrupt, with a rapid decrease in OD600 noted immediately following phage addition. This suggests a swift onset of phage activity. However, unlike in LB medium, the magnitude of bacterial growth suppression is less intense, and a plateau is reached relatively quickly. This plateau phase could suggest a dynamic equilibrium where the bactericidal action of the phage might be counterbalanced by bacterial regrowth or the potential onset of resistance mechanisms within the bacterial population. The differential responses observed could be attributed to the nutrient composition of the two media. The rich nutrient environment of LB medium may potentially support more vigorous phage replication and subsequent bacterial lysis. The phage-bacteria interactions observed in artificial urine may mirror the intricate dynamics within the urinary tract, akin to the comprehensive overview provided by Żaczek et al (Żaczek et al., 2020), highlighting the significance of comprehending phage-host interplay across different bodily fluids. Additionally, it is crucial to consider these findings in the context of studies like Zulk et al

(Zulk et al., 2022), which reveal the complexities of phage behavior in more natural environments such as human urine. Zulk et al. observed a reduction in phage lytic activity and the rapid emergence of phage-resistant bacterial strains in human urine. However, interestingly, these phage-resistant bacteria might be more susceptible to certain antibiotics, providing a theoretical basis for the synergistic use of phages and antibiotics, which could have significant clinical implications. The stability of P2-71 in artificial urine suggests its potential for long-term treatment of UTIs. This stability implies that P2-71 could remain effective in the complex environment of urine, providing a sustained therapeutic approach. This is particularly significant for UTI cases requiring long-term management, as the stable activity of the phage helps maintain consistent therapeutic effects over time. While our study demonstrates the stability of P2-71 in artificial urine, a notable limitation is the absence of investigation into bacterial resistance development against this phage. As underscored by Oromí-Bosch et al. (Oromí-Bosch et al., 2023), understanding and addressing bacterial resistance is crucial for the effective application of phage therapy. The potential for bacteria to develop resistance over time to P2-71, and the strategies to mitigate such resistance, remain unexplored in our research. Future studies are therefore essential to examine the resistance dynamics specific to P2-71 and to develop methods that could sustain its efficacy, such as phage cocktails, phage engineering, and phage-antibiotic synergy. Addressing these aspects is critical to ascertain the long-term clinical applicability of P2-71 in UTI treatment.

In summary, our study successfully isolated the lytic *P. mirabilis* phage P2-71 and provided a detailed description of its biological properties and genome. These findings not only offer valuable insights into phage therapy as a potential alternative to antibiotic treatments but also hold significant implications for the management of UTIs caused by MDR bacteria in human and veterinary healthcare.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number OR672055.

Ethics statement

The animal study was approved by The Sichuan Agricultural University's Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZD: Formal analysis, Investigation, Methodology, Writing – original draft. RW: Investigation, Writing – original draft. LL: Software, Writing – original draft. SA: Software, Writing –

original draft. JY: Data curation, Writing – original draft. QL: Funding acquisition, Resources, Supervision, Writing – original draft. KF: Funding acquisition, Resources, Supervision, Writing – original draft. YZ: Funding acquisition, Resources, Supervision, Writing – original draft. HF: Project administration, Validation, Writing – review & editing. ZYZ: Project administration, Validation, Writing – review & editing. HL: Project administration, Validation, Writing – review & editing. ZJZ: Project administration, Validation, Writing – review & editing. XQ: Conceptualization, Writing – review & editing. GP: Conceptualization, Methodology, 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/fcimb.2024.1347173/full#supplementary-material>

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Case report: Personalized triple phage-antibiotic combination therapy to rescue necrotizing fasciitis caused by Panton-Valentine leukocidin-producing MRSA in a 12-year-old boy

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Maximal standard-of-care (SOC) management could not stop the life-threatening progression of a necrotizing fasciitis induced by Panton-Valentine Leukocidin-producing Methicillin-Resistant *Staphylococcus aureus* (MRSA) in a 12-year-old boy. Multi-route phage therapy was initiated along with antibiotics against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, eventually leading to full recovery with no reported adverse events.

KEYWORDS

MRSA, Panton and Valentine leukocidin (PVL), bacteriophage, phage therapy, necrotizing fasciitis, pediatric intensive care unit

Introduction

S. aureus is an important pathogen of necrotizing fasciitis, and MRSA isolates in particular have been reported to feature increasing prevalence and high correlated mortality in these patients (Cheng et al., 2011; Tsai et al., 2021). Pantan-Valentine Leukocidin (PVL) is a cytotoxin produced by certain *S. aureus* strains, and is known to enhance *S. aureus*' virulence and necrosis-inducing potential; accordingly, necrotizing fasciitis resulting from PVL-*S. aureus* infection have been reported (Gillet, 2013; Hussain et al., 2022). In this case, PVL-MRSA-induced necrotizing fasciitis failed to respond to maximal SOC management: as a last resort therapy, it was treated by an exceptional multi-route phage therapy (PT) specifically targeting three involved bacterial species, in combination with antibiotics. This did not trigger any detected adverse event, and was followed by complete resolution of the infection.

Case description

A 12-year-old boy with no significant medical or surgical history was transferred (D0) to the Pediatric Intensive Care Unit (PICU) (Figure 1). On D-8, he suffered a minor trauma causing a sprained left ankle with superficial scratches. A plaster boot cast was set on D-7, but removed on D-6 because the patient reported increasing pain. On D-3, the child became confused and on D-1, febrile at 39°C. Clinical findings included tachycardia, polypnea, and swollen, painful, warm and red left leg and foot. Biological findings included signs of inflammatory syndrome (C-reactive protein 304 mg/L - normal value <5 mg/L) and multiple organ failure including respiratory and hemodynamic failure as well as liver, kidney and muscle damage, leading to PICU admission. Blood cultures were performed and empirical intravenous (IV) flucloxacillin, clindamycin and cefotaxime were initiated. Exceeding intramuscular pressure was measured in all eight tested compartments in the left foot and leg. Fasciotomies were performed, revealing pus in the soft tissues of the left lower limb.

Vasopressor therapy, sedation and endotracheal intubation were maintained upon return to PICU. Microbiological cultures of pus and blood grew for methicillin-resistant *Staphylococcus aureus* (MRSA) producing Pantan-Valentine Leukocidin (PVL). IV vancomycin was initiated on D+1, replacing cefotaxime and flucloxacillin, while clindamycin was pursued. Since we suspected significant contribution from the PVL toxin to the patient's condition, IV polyclonal immunoglobulins (1 g/kg) were administered on D+1.

Despite these adapted standard-of-care (SOC) measures and in-range therapeutic drug monitoring of vancomycin within 48h of initiation, the infection rapidly progressed to generalized necrotizing fasciitis and pyomyositis on D+2, as confirmed by full-body MRI (Figure 1). This justified extended fasciectomy and debridement on multiple sites on D+8. Ascites appeared and was drained on D+2. Thoracic CT-scan revealed disseminated septic emboli in both lungs on D+1. Chest X-ray showed a right pleural effusion on D+8, which was drained with purulent discharge confirming empyema. Microbiological cultures of both the peritoneal and pleural fluids grew for the same MRSA-PVL strain.

Diagnosis, therapeutic measures, outcomes

Between D0 and D+8, sustained MRSA-PVL bacteremia was observed and the patient's state was significantly deteriorating to a life-threatening situation, despite maximal SOC management. Necrotizing fasciitis diagnosis was assessed based on magnetic resonance imaging (MRI) findings (Figure 2), microbiological culture findings (*S. aureus*-positive pus and blood cultures), and aforementioned evidence of compartment syndrome by multiple compartment pressure measurements. *S. aureus*-positive blood cultures combined with persistent need for vasopressor therapy defined the complementary diagnosis of septic shock syndrome.

As rescue therapy, phage therapy (PT) was initiated on D+9. PT is the use of specific bacteriophage viruses ("phages"), natural viruses that exclusively infect and lyse a specific bacterial genre or species, as bactericidal therapeutic agents. A solution containing the anti-*S. aureus* phage named ISP was obtained from Queen Astrid Military Hospital ([QAMH], Brussels, Belgium) after on-plate phage susceptibility testing ("phagogram") revealed that phage ISP had potent lytic power against the patient's MRSA-PVL strain (EOP = 1, i.e. "efficiency of plating", a phage's relative lytic efficiency on a clinical strain compared to the phage's reference host strain, expressed as a ratio of the number of plaques theoretically ranging from 0 to 1). The initial ISP phage solution, of titer 10^9 plaque forming units (PFU)/mL, was diluted 100 times in NaCl 0.9% to reach a therapeutic titer of 10^7 PFU/mL. Multi-route PT was then initiated using this 10^7 PFU/mL solution (Figure 1). IV PT was administered at 50mL over 6h every 24h during 22 days (D+9 to D+30). Intra-pleural and intra-peritoneal PT were administered through the respective drainage catheters: once daily, cavities were rinsed with 5% sodium bicarbonate solution for 15 minutes, before 40mL of phage solution were instilled. Catheters were then clamped for 20 minutes before being reopened, without aspiration. Topical PT was applied daily on open wounds with dressings soaked in

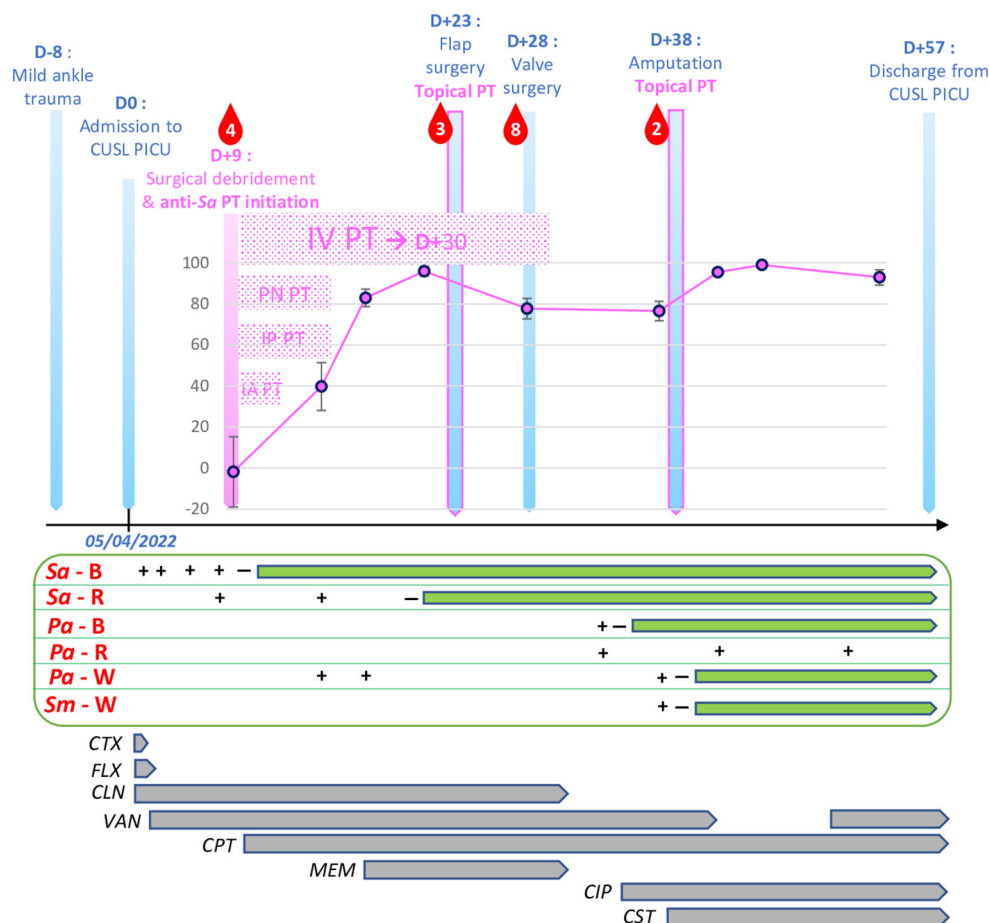


FIGURE 1

Timeline of the clinical case's main events. Main medico-surgical events are represented as blue vertical arrows. Phage therapy-related events are represented in pink. Pink curve represents degree of Phage Immune Neutralization (PIN) against phage ISP in % of neutralized titer at 30 minutes. Error bars represent standard deviation of the means. Red droplets represent units (205 mL) of blood transfused. D, day; Sa, *Staphylococcus aureus*; Pa, *Pseudomonas aeruginosa*; Sm, *Stenotrophomonas maltophilia*; B, Blood culture; R, Respiratory tract-originated sample culture (broncho-alveolar lavage or endotracheal aspirate); W, wound sample culture (swab or intraoperative biopsy); PT, phage therapy; IV, intravenous; PN, pulmonary nebulization; IP, intra-pleural; IA, intra-abdominal; CTX, Cefotaxime; FLX, Flucloxacillin; CLN, Clindamycin; VAN, Vancomycin; CPT, Cefepime; MEM, Meropenem; CIP, Ciprofloxacin; CST, Colistin.

phage solution for 20 minutes. Lastly, bronchopulmonary PT was administered by mesh-nebulization of 4 mL of phage solution at the higher titer of 10^8 PFU/mL. IV ceftazidime was also added to the treatment eight hours after the first IV PT dose. Additionally, following isolation of *Pseudomonas aeruginosa* in several wounds on D+14, IV meropenem was administered from D+17 until D+30 (Figure 1).

Objective improvements were rapidly noticed. The first blood culture performed after PT initiation and ceftazidime initiation (sampled on D+10, i.e. 30h after PT initiation and 22h after ceftazidime initiation) did not grow for MRSA-PVL, nor did any ulterior blood culture. Peritoneal and pleural catheters were withdrawn after three and seven days of PT respectively. Despite these improvements, the evolution remained complicated, with extubation failure related to persistent lung damage and bacterial tracheitis, and mitral valve abscess related to sustained MRSA-PVL bacteremia, cured by open-heart valve repair surgery on D+28. Septic necrosis led to extensive tissue loss in the left foot and leg, with no hope for spontaneous wound closure. Reconstruction by flap surgery using right latissimus dorsi was attempted on D+23.

Intra-operative topical PT was applied using ISP solution (10^7 PFU/mL) for syringe rinsing of the surgical site and soaking of dressings applied for 45 minutes. However, flap necrosis started on D+32, eventually leading to transtibial amputation of the left leg on D+38. Infection of the left leg wounds by *Stenotrophomonas maltophilia* and *P. aeruginosa* had been diagnosed on D+37. Accordingly, we administered intra-operative topical PT on the leg stump before surgical closure, applying soaked dressings and rinsing with 200 mL of a 10^7 PFU/mL mix of anti-*S. maltophilia* phage BUCT700 and anti-*P. aeruginosa* phages PNM, 14-1 and PT07. Like anti-*S. aureus* phage ISP, these anti-*S. maltophilia* and anti-*P. aeruginosa* phages were also selected based on favorable phage susceptibility testing ("phagogram") results on the respective patient's bacterial isolates.

After amputation, sustained favorable clinical progression was observed along durable eradication of all *S. aureus*, *S. maltophilia* and *P. aeruginosa* from the bloodstream and all wounds. Besides amputation, the child progressively reached full recovery of all wounds and of respiratory function. The child was transferred from PICU to pediatric hospitalization unit on D+57, then to



FIGURE 2

Full body MRI, coronal view. Short-T1 inversion recovery (STIR) mode. Image shows diffuse hyperintense inflammatory infiltration in subcutaneous soft tissues of both lower limbs, the upper left limb, the thorax and the abdomen, as well as disseminated inflammatory pulmonary lesions and two inflammatory fluid collections in the right thigh and left shoulder (red arrows).

rehabilitation unit on D+104. Final discharge from hospital occurred on D+145. One year after hospital discharge, the patient remains cured of all infections. Stump osteitis was suspected on clinical basis 5 months after hospital discharge, and was successfully

treated by surgical revision along with vancomycin and clindamycin administration. Functional rehabilitation and adaptation to the prosthetic leg appear successful. Cardiological follow-up upon initial hospital discharge diagnosed ectopic atrial tachycardia, likely linked to previous bacterial valve abscess and related surgery. This was initially treated by flecainide, which was successfully withdrawn after 4 months without needing further re-introduction.

Discussion

This severe infection required an exceptional multiple bacteriophage-antibiotic combination therapy directed against three pathogens: *S. aureus*, *P. aeruginosa* and *S. maltophilia*. This is likely the first report ever of a patient being treated by three distinct documented phage therapies, targeting three distinct pathogens, all with their dedicated route(s) of administration and combined antibiotic therapy. Sustained MRSA bacteremia and its associated metastatic sites of infection are a known therapeutic challenge: seemingly appropriate SOC management often fails to eradicate bacteremia, and PT is increasingly reported as a potentially promising complementary approach (Holland et al., 2022). In this case, PT initiation was followed by the eradication of these three pathogens in their respectively treated compartments. Yet, several aspects of the clinical and microbiological progression deserved further attention.

The occurrence of Phage Immune Neutralization (PIN), the development of an antibody response against administered phages, was investigated using modified Adams protocol by double agar overlay assay on numerous serum samples collected before, during and after PT (Adams, 1959). PIN occurred against phage ISP, starting six days after PT initiation (D+14) (Figure 1). Transient decrease in PIN magnitude between D+21 and D+41 is likely due to hemodilution by numerous blood transfusions (Figure 1) and extracorporeal circulation during open-heart surgery. Consistent with previous studies, this potent and early-onset PIN was not followed by therapeutic failure, though it might mitigate the efficacy of ulterior reintroduction of phage ISP (Łusiak-Szelachowska et al., 2017; Dedrick et al., 2021).

Evaluation of PT's net contribution to *S. aureus*' eradication from the bloodstream is a limitation to this work. It is indeed difficult because of the initiation of ceftaroline eight hours after PT initiation and the absence of blood cultures that could have been collected in this short interval between PT initiation and ceftaroline initiation.

Nevertheless, using an OmniLog[®] automated incubator, we investigated and compared in 96-wells microplates triplicates the *in vitro* bacterial growth kinetics of the patient's *S. aureus* strain subjected to various combinations of different titers of phage ISP, ceftaroline, and the combination of both, as already reported in similar works (Van Nieuwenhuyse et al., 2021, 2022). The results from this assay suggest, at least at certain phage and antibiotic titers, that their combined use is able to durably suppress bacterial growth in a synergistic fashion (Figure 3). The translatability of

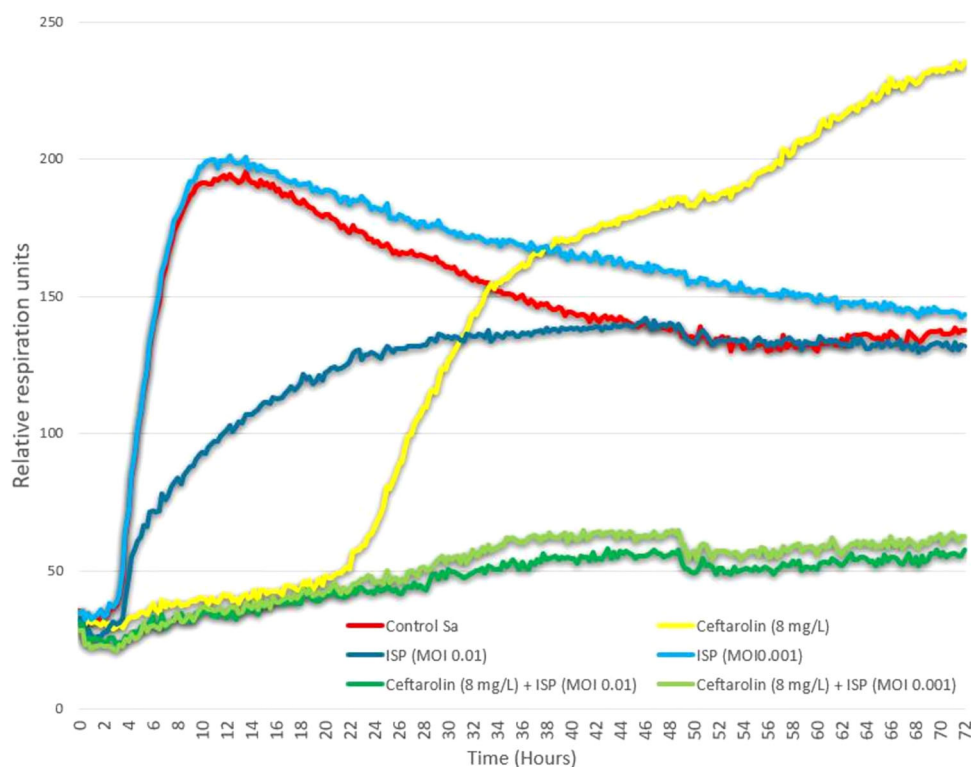


FIGURE 3

Phage-antibiotic interaction testing. Activities of phage ISP (at multiplicity of infection [MOI] = 0.01 and 0.001) and ceftaroline (at 8mg/L) as well as the combinations of these phage titers with this ceftaroline concentration, were determined using an OmniLog® automated incubator (Biolog, Hayward, CA, USA). Data was analyzed with OmniLog data Analysis Software (v1.7). Experiments were done in 96-well plates in a final volume of 200 μ l of LB supplemented with 100 times diluted tetrazolium dye mix, according to the manufacturer's instructions. Bacterial cells were added at a concentration of 105 colony forming units(cfu)/well, calculated based on optical density (OD, at 600 nm) measurements (with an OD of 0.5 corresponding to 4×10^8 cfu/ml, on average), which were validated using a classical plate culture method. Plates were incubated at 37 °C for 72 hours and a possible reduction (causing a color change) of the tetrazolium dye due to bacterial respiration (during growth) was monitored and recorded every 15 min by the Omnilog system. Bacterial proliferation is presented through relative units of cellular respiration: efficacious phages, antibiotics, and combinations thereof, suppress bacterial proliferation. "Control Sa" curve represent control growth of a triplicate containing only the aforementioned bacterial load (*S. aureus*), with no antibiotic or phage. Results are presented as mean values of biological triplicates. While ceftaroline alone at 8mg/L and phage ISP alone at two different MOIs appear unable to durably suppress bacterial growth over the 72 hours of growth analysis, combination of this ceftaroline concentration with either of these two sub-inhibitory phage titers leads to durable suppression of bacterial growth, suggesting in vitro synergistic properties.

these *in vitro* properties to *in vivo* conditions, however, warrants further research.

PVL is a cytotoxin produced by various *S. aureus* strains, both methicillin-sensitive *S. aureus* (MSSA) and community-acquired methicillin-resistant *S. aureus* (CA-MRSA) (Shallcross et al., 2013). It is known to enhance *S. aureus*' virulence and necrosis-inducing potential (Gillet, 2013).

Some observations in our work correlated with features typically described in PVL-*S. aureus* infections: first, our patient had a severe infection leading to PICU as a result of hemodynamic and respiratory failure from a septic shock. In a prospective European multicentric study, 17% of children with community-acquired *S. aureus* (CA-SA) invasive infections had severe infection leading to death or admission to PICU, caused by both CA-MSSA and CA-MRSA strains (Gijón et al., 2016). The prevalence of PVL-positive CA-SA invasive infections amounted to 18.6%, and only 7.8% of the isolates were MRSA. In this study, PVL expression is a factor independently associated with outcome severity, regardless of methicillin resistance (Gijón et al., 2016).

Second, PVL-associated bone and joint infections (BJI) in children are correlated with higher biological inflammatory markers at presentation, a longer course of illness, more febrile days, more complicated/severe infection (muscle abscess, pyomyositis, subperiosteal abscess, visceral abscess, and deep venous thrombosis) and more intensive care unit admissions, according to a systematic review including 15 studies on children suffering from such BJI (Bouiller and David, 2023). These typical features are again in line with our report.

Third, PVL-associated community-acquired pneumonia (CAP) and its severity are not evenly distributed according to age. Gillet and colleagues described two distinct entities of PVL-associated staphylococcal CAP with differences regarding clinical presentation and outcome: staphylococcal CAP in toddlers (i.e. pleuropneumonia) and staphylococcal necrotizing pneumonia in young adults. In toddlers, PVL-negative CAP is virtually absent, presentation matches with pleuropneumonia, SOC appears to be sufficient and overall mortality is low (Gillet, 2013; Gillet et al., 2021). Contrastingly, necrotizing PVL-associated CAP in

adolescents and adults is associated with specific symptoms at admission as cutaneous rash, airway hemorrhage and leukopenia. It occurs mostly in younger, previously healthy adults, and is often preceded by influenza-like symptoms. Its outcomes are extremely severe with rapid onset of acute respiratory distress syndrome (ARDS) despite aggressive management, accounting for high mortality (39%) (Sicot et al., 2013; Gillet et al., 2021). In our case, despite PVL status and absence of underlying conditions, the entity of staphylococcal necrotizing pneumonia was not entirely congruent because the patient showed no airway bleeding, no leukopenia, and had a delayed onset of pneumonia. A secondary pulmonary involvement due to secondary hematogenous infection and septic pulmonary emboli seems more likely, as opposed to an inhalation-mediated infection leading to the aforementioned typical necrotizing phenotype.

Lastly, sub-MIC titers of β -lactams and, to a lesser extent, vancomycin may enhance bacterial production of PVL, but this over-expression can be blocked by the combined use of an antitoxin agent (Dumitrescu et al., 2008). As such, the concomitant use of clindamycin, rifampin or linezolid is thus recommended in clinical practice (Dumitrescu et al., 2008). Nevertheless, antibiotics alone often fail to fully contain such infections, as antibiotic distribution is reduced in necrotic tissue. Therefore, early and complete drainage of all PVL suppuration and debridement of necrotic tissues by extensive and, if needed, repeated surgeries is mandatory whenever feasible and as soon as possible (Gillet et al., 2011). This additional need for surgical management in bone and joint infections and complicated skin and soft-tissue infections is again consistent with our case.

It should also be noted that further PCR-based typing of the *S. aureus* strain revealed the absence of expression of exfoliative toxins ETA and ETB and of toxic shock syndrome toxin 1 (TSST-1) (Table 1). Protein A (spa)-typing linked the strain to type t5691, related to multilocus sequence type ST152 (Lebughe et al., 2017). ST152 is a known community-acquired MRSA-PVL. Its spread in both Europe and sub-Saharan Africa is consistent with the patient's history (Baig et al., 2020). However, ST152 is not known as an intrinsically virulent clone (Baig et al., 2020). The triggering of such a hyper-virulent disseminated infection by a non-hyper-virulent MRSA-PVL strain led to the research of specific risk factors, including inborn errors of immunity (IEI) (Gillet, 2013; Bousfiha et al., 2022). Routine immunological work-up showed no abnormal values in blood count except for low B-cells (143/ μ L [N: 200-600/ μ L]), more marked in the B-memory cells (Total B-memory CD27+ cells: 14/ μ L [N: 50-200/ μ L] – relative abundance 9.8% [N: 13.3-47.9%]). Whole-exome sequencing provided no molecular basis allowing for the diagnosis of IEI, and specifically no heterozygous defects in OTULIN, which are known to expose carriers to severe necrotic staphylococcal infections by dysregulating nonhematopoietic cells' response to alpha-toxin, a major staphylococcal virulence factor (Spaan et al., 2022) (Supplementary Tables 1 and 2).

Whole-genome sequencing of the initially retrieved *S. aureus* isolate was performed to refine resistance and virulence typing. Genomic DNA was extracted (DNeasy UltraClean Microbial kit, Qiagen) and prepared for Nextera Flex (Illumina) and sequenced on an Illumina Miniseq machine using a paired-end approach (2*150 bp). The quality of the sequencing data was assessed (Fast QC, Galaxy Version 0.12.1) and trimmed appropriately (Trimmomatic

TABLE 1 Strain typing.

S. aureus	typing	S. aureus	AST		P. aeruginosa	AST	S. maltophilia	AST
		antibiotic	MIC		antibiotic	interp.	antibiotic	interp.
PVL	positive	Amikacin	8	R	Amikacin	S	Amikacin	R
TSST-1	negative	Ceftaroline	1	S	Aztreonam	S	Aztreonam	R
ETA	negative	Clindamycin	<0.25	S	Ceftazidim	S	Ceftazidim	R
ETB	negative	Ciprofloxacin	<0.25	S	Ciprofloxacin	S	Ciprofloxacin	S
spa-type	t5691	Cefoxitin	>8	R	Colistin	S	Cefepim	R
MST type	ST152	Gentamycin	>4	R	Cefepim	S	Gentamycin	R
		Moxifloxacin	<0.25	S	Imipenem	S	Imipenem	R
		Oxacillin	>2	R	Minocyclin	R	Minocyclin	S
		Rifampin	<0.25	S	Meropenem	S	Meropenem	R
		TMP-SMX	>4/76	R	Piperacillin-Taz.	S	Piperacillin-Taz.	R
		Teicoplanin	<1	S			TMP-SMX	S
		Tetracyclin	<0.5	S				
		Vancomycin	1	S				

Antibiotic susceptibility testing (AST) data are presented for an array of tested antibiotics for aforementioned isolates of *S. aureus*, *P. aeruginosa* and *S. maltophilia*. For *S. aureus*, minimum inhibitory concentrations (MIC) were assessed by standard microdilution method, expressed in mg/L. AST is assessed by standard disk diffusion method for *P. aeruginosa* and *S. maltophilia*. Interpretation (interp.) finds the respective isolates susceptible (S) or resistant (R) to the tested antibiotic. Further characterization of typical virulence factors of *S. aureus* is performed by polymerase chain reaction (PCR) and illustrates expression of PVL (Panton-Valentine Leukocidin) by the isolate, but no expression of TSST-1 (toxic shock syndrome toxin 1) or exfoliative toxins A or B (ETA, ETB). Further protein- and genome-based characterization of the *S. aureus* isolate links it to spa-type t5691, related to multilocus sequence type ST152. TMP-SMX, trimethoprim-sulfamethoxazole; piperacillin-taz., piperacillin-tazobactam.

(Bolger et al., 2014), Galaxy Version 0.39). Genomes were constructed using Unicycler (Galaxy Version 0.5.0) (Wick et al., 2017). Genome annotation was performed using Prokka (Galaxy Version 1.14.6) (Seemann, 2014). Further function annotation was performed using Abriicate (Galaxy Version 1.0.1) (Seemann, 2017) through ARG-ANNOT (ARG-ANNOT NT v.6, July 2019), CARD (v.3.1.4 to 3.2.5), ResFinder, https://bitbucket.org/genomicepidemiology/resfinder_db for antibiotic resistance genes and VirulenceFinder 2.05.6 database for virulence genes (accessed on July 8, 2024) (Joensen et al., 2014; Tetzschner et al., 2020). The genomes were scanned against PubMLST schemes using MLST7 (Galaxy Version 2.22.0).

Sequencing product is available on NCBI BioProject with accession number JBFQYI000000000. Results confirmed the findings from both the former first-line molecular typing and the antibiotic susceptibility testing. Indeed, regarding antimicrobial resistance, presence of genes *dfrG*, *blaZ*, and *aac(6')-aph(2'')* were phenotypically correlated to aforementioned resistance to trimethoprim-sulfamethoxazole, (amino) penicillins and aminosides respectively, while *mecA* was responsible for the identified MRSA phenotype and its associated resistance to all tested Beta-lactam antibiotics besides ceftaroline. These sequencing products were used in SCCmecFinder (version 1.2) to predict the strain's SCCmec type. No whole SCCmec cassette was predicted with a template coverage threshold of at least 40%. Nevertheless, SCCmec type XIII (9A) was considered the most likely prediction based on *mec* class A and *ccr* class 9 (93.1% identity query with *ccrC2* allele 1:1:KR187111), SCCmec type XIII having been first characterized in 2018 based on another ST152 strain (Baig et al., 2018). Results regarding virulence confirmed the presence of genes *lukF-PV* and *lukS-PV* coding for Panton Valentine leukocidin F & S components respectively, and initial annotation attempt did not detect any known enterotoxin or exfoliatin present in the used database. These synthetic results are made available as [Supplementary Material](#). However, a specific BLAST confirmed that this strain expressed a variant of exfoliative toxin E (coded by *ete* gene) with 100% homology to an *ete* variant, called *ete2*, recently described in a case report which was interestingly also focusing on a PVL-positive clonal complex 152 *S. aureus*-related necrotizing fasciitis (Sabat et al., 2022). In this work, the authors also point out that *ete2* variant has been described exclusively in clonal complex 152 *S. aureus* so far. The net contribution of *ete2* to the virulent phenotype encountered in these necrotizing fasciitis cases is still unclear, and warrants further research. These synthetic results are made available as [Supplementary Table 3](#).

Patient perspective

Adolescents may be able to make medical decisions on their own, even when the choice and its implications are particularly challenging. However, in this case, direct discussion with the patient at the most critical time during the course of events was not possible due to sedation and intubation during the septic shock management. It was thus not possible to define if he was mature enough to understand the situation in full. Subsequently, exposing the

full complexity of the situation to his parents, and more specifically convincing them eventually that transtibial amputation was essential to save their son's life was no easy task. It required sustained day-to-day communication about this complex situation, making sure every aspect of it was understandable while avoiding the pitfall of oversimplification; most of all, it mostly required deep empathy at all time. Despite the heartbreaking nature of this decision, the parents eventually consented to the transtibial amputation, convinced it was indeed the only way to stop further progression of this life-threatening infection.

Data availability statement

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

Ethics statement

For the approval of the study regarding genome sequencing (SAID-WES protocol): Comité d'Éthique Hospitalo-Facultaire Cliniques universitaires Saint-Luc UCLouvain. Regarding the case in itself and consent to publish: written consent obtained but no Ethics Committee approval considered necessary since this is a case report of a patient where nothing outside of the locally-defined standard-of-care was applied. Prior authorization to allow by default the publication of such retrospective case reports of hospitalized patients. Positive opinion of this general practice emitted by the related ethics committee: Comité d'Éthique Hospitalo-Facultaire Cliniques universitaires Saint-Luc UCLouvain. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

BV: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft. MB: Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Data curation. OC: Writing – review & editing. AH: Writing – review & editing. ED: Writing – review & editing. TD: Writing – review & editing. SC: Funding acquisition, Resources, Writing – review & editing. CB: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – review & editing. LB: Writing – review & editing. J-CY: Writing – review & editing. JD: Conceptualization, Writing – review & editing. OC: Writing –

review & editing. P-LD: Writing – review & editing. AL: Writing – review & editing. RM: Visualization, Writing – review & editing. HR-V: Writing – review & editing. AV: Writing – review & editing. SD: Writing – review & editing. MM: Investigation, Methodology, Resources, Supervision, Writing – review & editing. JG: Investigation, Methodology, Resources, Writing – review & editing. J-PP: Resources, Supervision, Writing – review & editing. LH: Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing. DV: Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2024.1354681/full#supplementary-material>

SUPPLEMENTARY TABLE 1

Patient genome - sequencing findings in the field of OTULIN & OTULINL genes modifications.

SUPPLEMENTARY TABLE 2

Patient genome - whole-exome sequencing and annotation data.

SUPPLEMENTARY TABLE 3

Strain genome - sequencing and annotation data in the field of antimicrobial resistance and virulence determinants.

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