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Food-Derived Alkaloid Isoliensinine Mitigates *Staphylococcus aureus*-Triggered Joint Inflammatory Response and Tissue Damage

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ABSTRACT: Isoliensinine, a bisbenzylisoquinoline alkaloid derived from the embryo of *Nelumbo nucifera* (lotus seed), is widely used in functional foods and traditional herbal infusions because of its anti-inflammatory and antioxidant properties. In this study, we explored its potential as a food-derived antivirulence agent against methicillin-resistant *Staphylococcus aureus* (MRSA), a clinically significant pathogen responsible for severe infections, including septic arthritis. Although MRSA is associated primarily with healthcare- and community-acquired infections, its occasional detection in food products such as meat and dairy raises public health concerns regarding its broader ecological distribution. *In vitro*, isoliensinine markedly suppressed MRSA cytotoxicity by inhibiting hemolysin activity, reducing bacterial adhesion and invasion, and downregulating key virulence gene expression without affecting bacterial growth—thus avoiding selective pressure for resistance. Mechanistic investigations via a cellular thermal shift assay (CETSA), surface plasmon resonance (SPR), and electrophoretic mobility shift assay (EMSA) confirmed that isoliensinine directly binds to SaeR, the response regulator of the SaeRS two-component system, and blocks its DNA-binding activity, thereby impairing the transcriptional control of multiple virulence determinants. *In vivo* efficacy was demonstrated in both *Galleria mellonella* and a rat model of MRSA-induced septic arthritis. Isoliensinine treatment significantly reduced the bacterial burden, alleviated synovial inflammation, and preserved cartilage and bone structure, indicating strong protective effects against infection-associated joint damage. Given its natural dietary origin, safety profile, and mechanistic specificity, isoliensinine has strong potential for development as a next-generation nutraceutical or functional food additive aimed at preventing or mitigating infections associated with virulent pathogens.

Keywords: Isoliensinine; SaeRS two-component system; Septic arthritis; Methicillin-resistant *Staphylococcus aureus*

1. Introduction

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The lotus seed embryo (*Nelumbo nucifera* Gaertn), commonly referred to as *lian zi xin*, is a traditional food ingredient widely used in East Asia and Southeast Asia. In addition to its culinary applications, *lian zi xin* has long been regarded in traditional Chinese medicine as a functional food with calming, anti-inflammatory, and cardiovascular benefits^[1-3]. Modern phytochemical studies have identified the presence of bisbenzylisoquinoline alkaloids—such as neferine^[4], liensinine^[5], and isoliensinine (IL)—as major active constituents, which contribute to their antioxidative, antihypertensive, and lipid-regulating properties. These compounds have garnered increasing attention for their potential role in promoting human health through dietary or nutraceutical interventions.

Recent public health concerns have highlighted the dual threat posed by *Staphylococcus aureus*, particularly methicillin-resistant strains (MRSA), not only as a leading cause of hospital- and community-acquired infections but also as an emerging foodborne risk. MRSA has been intermittently detected in various food products, including raw meat and dairy, indicating its potential entry into the human food system through food production, processing, and distribution channels. This raises serious concerns about its possible transmission through the food chain and its implications for food safety and human health^[6-8]. Furthermore, genetic analysis of foodborne MRSA strains revealed that certain lineages (such as strains ST398 and ST9) possess cross-host transmission capabilities, enabling gene exchange and infection transmission between animals and humans^[9-11]. This phenomenon is more prevalent than in *Pseudomonas aeruginosa*^[12]. These characteristics position MRSA not only as a “superbug” within healthcare systems but also as a high-risk pathogen lurking within food safety systems, posing severe challenges to public health.

Among the serious clinical outcomes associated with MRSA, septic arthritis stands out as a particularly debilitating manifestation, especially when the pathogen infiltrates bone and joint tissues. This infection often arises via hematogenous spread but may also result from direct inoculation due to trauma or from the contiguous extension of nearby infections^[13, 14]. Notably, the persistent and destructive course of MRSA-induced joint infections is closely linked to the ability of bacteria to sense host environmental cues and adaptively regulate virulence gene expression.

In response to host environmental cues, SaeS activates SaeR, which subsequently increases the production of toxins (e.g., alpha-hemolysin^[15]), immune evasion factors, and adhesion proteins crucial for bacterial colonization of host tissue^[16, 17]. Unlike the Agr system, whose activity often varies across strains and can be diminished under glucose-rich or low-oxygen conditions, SaeRS is robustly triggered by neutrophil- and blood-derived cues, ensuring timely induction of virulence during early host contact^[16, 17]. For example, SaeR activation significantly enhances the expression of toxins such as Hla, as well as adhesion factors such as the clumping factors ClfA and ClfB and the fibronectin-binding proteins FnBPA and FnBPB, all of which play pivotal roles in facilitating bacterial adherence to synovial tissues and resisting phagocytosis^[18-20]. This coordinated control couples cytotoxicity with adherence, providing a single, host-responsive regulatory choke point that directly links the sensing of host signals to the expression of factors required for invasion and immune evasion. Moreover, studies indicate that SaeR/S-regulated immune evasion factors, such as

chemotaxis inhibitory protein (CHIP) and staphylococcal complement inhibitor (SCIN), interfere with host immune recruitment and complement activation^[17]. Consistent with this central role, genetic disruption or pharmacologic inhibition of SaeRS diminishes toxin output and attenuates disease severity in skin, pneumonia, and bacteremia models^[21]. Other global regulators are less attractive for complementary reasons. SarA broadly affects adhesion/biofilm and multiple loci but has pleiotropic, network-embedded effects that are less toxin-centric than those of SaeRS^[22]. CodY/CcpA integrate a metabolic state with virulence; perturbing them risks fitness costs akin to growth inhibition (undermining an antivirulence rationale)^[23]. In comparison, SaeRS offers a more direct, conserved, and host-synchronized choke point over the cytotoxic output without necessarily imposing strong bactericidal selection.

In this study, we screened bioactive alkaloids from lotus seed embryos to identify molecules with protective effects against MRSA-induced inflammatory stress. Isoliensinine has emerged as the most promising candidate. We then assessed its impact on MRSA virulence regulation and its tissue-protective effects in cell and animal models of MRSA joint infection. Overall, this work highlights the potential of food-derived natural compounds to alleviate infection-associated inflammation.

2. Materials and methods

2.1 Bacterial strains and reagents

The MRSA USA300 strain used in this study is a reference community-associated MRSA isolate acquired from the American Type Culture Collection (ATCC; Manassas, VA) and therefore represents a well-characterized laboratory strain used broadly for mechanistic and virulence studies. The *S. aureus* USA300 SaeR-knockdown strain (*kd-SaeR*)^[24], the pET28a-saeR plasmid, and the bioluminescent USA300 *LAC::lux* strain were developed in our laboratory. Detailed strain information is provided in the Supplementary Table 1. *Escherichia coli* (*E. coli*) DH5 α was used as a host for vector construction. *S. aureus* and *E. coli* were cultured in brain heart infusion (BHI, Hopebio, China) and Luria–Bertani (LB, Hopebio, China) broth, respectively, at 37°C with shaking at 220 rpm. Kanamycin (100 μ g/mL) was added to the media when necessary for plasmid selection or maintenance. Isoliensinine, a natural product with a purity of 99.7%, was supplied by Desite Biotechnology Co., Ltd. (Chengdu, China). The quality report for isoliensinine is shown in the Supplementary Figure 2. The general reagents used were purchased from Sangon Biotech (Shanghai, China).

2.2 Thermal shift assays

The TSA experiments were performed via a Bio-Rad iQ5 (Bio-Rad, USA) with SYPRO Orange (Sigma–Aldrich, USA) in a 96-well plate. SaeR protein (final concentration, 2 μ M) was mixed with TSA buffer (150 mM NaCl, 10 mM HEPES, pH 7.5) in the presence of SYPRO Orange dye^[25]. In one experimental setup, ten different compounds were added to the SaeR protein mixture to evaluate their effects. The compounds used and their concentrations are listed in Supplementary Table 2. The plate was heated from 25°C to 90°C at a rate of 1°C/min, and the fluorescence was measured (excitation: 475 nm; emission: 590 nm).

2.3 Minimum inhibitory concentration (MIC) determination

The MIC of the isoliensinine against *S. aureus* USA300 was determined via the broth microdilution method. The bacterial cultures were diluted 1:1000 in fresh cation-adjusted Mueller–Hinton broth (CAMHB) and grown to the logarithmic phase^[26, 27]. In a 96-well plate, 100 μ L of CAMHB and *S. aureus* USA300 (1×10^5 CFU) were mixed with serial dilutions of isoliensinine (0–256 μ g/mL). After 16 hours of incubation at 37°C, the MIC was determined as the lowest concentration at which no visible bacterial growth occurred.

2.4 RNA extraction and RT–qPCR analysis of *S. aureus* virulence factors

S. aureus was grown in BHI medium to an OD₆₀₀ of 0.3. At this point, isoliensinine was added to the culture at a final concentration of 64 μ g/mL, and the incubation continued at 37°C for 24 hours. The control groups received the same concentration of DMSO (0.1%). After incubation, total RNA was extracted from the bacterial culture via the TRIzol method^[28]. The synthesis of complementary DNA (cDNA) was performed via the PrimeScript RT Reagent Kit (TaKaRa, China), and RT–qPCR was conducted on an ABI 7900HT real-time PCR system. The relative expression levels of the target transcripts were normalized to those of *gyrB* as a reference gene. The primers used for RT–qPCR are listed in Supplementary Table 3.

2.5 Construction of *SaeR* and its Mutant Plasmids

The DNA sequence encoding *SaeR* was amplified from the genomic DNA of *S. aureus* USA300 via PCR. The PCR product was digested with XhoI and BamHI and then cloned and inserted into the pET28a vector. Sequence verification of the cloned fragment was conducted via DNA sequencing, resulting in the pET28a-*saeR* plasmid^[29]. Site-directed mutagenesis of TYR-186 and ARG-199 was performed via the Mut Express II Fast Mutagenesis Kit V2 (Vazyme Biotech, China), and the sequences of the primers used for the generation of these mutants are listed in Supplementary Table 3.

2.6 Expression and purification of *SaeR* and its mutants

Recombinant pET28a-*saeR* plasmids and their mutants were transformed into *E. coli* BL21 (DE3) via the heat shock method. The bacteria were cultured to an OD₆₀₀ of 0.6 and induced with 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 16°C for 16 hours^[30]. Proteins were purified via Ni-NTA resin (Beyotime Biotechnology, Shanghai, China) due to the presence of a 6-His tag in the recombinant pET28a-*saeR* plasmid. Nonspecific proteins were eluted with 50 mM imidazole, and purified proteins were collected with 400 mM imidazole.

2.7 Growth Curve Assay

Overnight cultures of *S. aureus* USA300 were diluted 1:100 in fresh BHI medium with or without 64 μ g/mL isoliensinine and grown to an OD₆₀₀ of 0.3. The cultures were divided into three groups: control (WT-DMSO), drug-treated (WT-isoliensinine), and kd-*SaeR*. The OD₆₀₀ nm absorbance was measured at 1-hour intervals over 24 hours via a UV spectrophotometer, and growth curves were plotted.

2.8 Cell viability assay

Chondrogenic cell lines derived from teratocarcinoma (ATDC5) or A549 cells (2×10^4 cells per well) were seeded in 96-well plates and cultured for 24 hours at 37°C in a humidified atmosphere containing 5%

CO₂. The cells were subsequently exposed to various concentrations of isoliensinine (0–64 µg/mL) for an additional 24 hours^[31]. Following treatment, the culture medium was removed, and 0.5 mg/mL MTT solution was added to each well and incubated for 4 hours to allow for formazan formation. The resulting crystals were dissolved in DMSO, and the absorbance was measured at 490 nm via a microplate reader.

2.9 Hemolysis Assay

Rabbit erythrocytes were collected by centrifugation at 4000 rpm for 3 minutes at 4°C, washed with PBS until the supernatant was clear, and resuspended in 0.5 mL of PBS. Different concentrations of isoliensinine (8–128 µg/mL) were added to the erythrocyte suspension, with PBS and Triton X-100 serving as negative and positive controls, respectively^[32]. After incubation at 37°C, the supernatant was collected, and the absorbance was measured at 600 nm.

2.10 Western blot analysis

S. aureus cultures were grown to an OD₆₀₀ of 0.3 and treated with isoliensinine (8–128 µg/mL) until the late logarithmic phase. Total protein was extracted via lysis buffer and quantified via a BCA protein assay kit (Thermo Fisher Scientific, USA). Proteins (40 µg) were separated by SDS–PAGE and transferred to PVDF membranes. After blocking with 5% nonfat milk, the membranes were incubated overnight at 4°C with a rabbit anti- α -hemolysin antibody (Sigma, USA) or a rabbit anti-LukS-PVL polyclonal antibody (Abcam, UK) at a 1:500 dilution. The secondary antibody used was HRP-conjugated goat anti-rabbit IgG (Beyotime Biotechnology, China)^[33]. Visualization was performed via an enhanced chemiluminescence kit (Merck Millipore, Germany), and imaging was performed via a chemiluminescence detection system.

2.11 Hemolysis Assay in Blood Agar Plates

The effect of isoliensinine on α -hemolysin activity was further assessed via blood agar plates. *S. aureus* cultures were grown to the logarithmic phase and adjusted to an OD₆₀₀ of 0.4. The supernatants were collected via centrifugation (5500 × g, 4°C, 3 min) and mixed with different concentrations of isoliensinine (16, 32, and 64 µg/mL) or an equivalent volume of DMSO^[34]. The mixtures were added to wells in blood agar plates, air-dried, and incubated at 37°C for 18–24 hours. Hemolytic zones were observed and quantified.

2.12 Fibronectin-Binding Assay

Bovine fibrinogen (20 mg/mL) was coated onto 96-well plates at 4°C overnight. After washing with PBS, 100 µL of 1% BSA was added to block the wells for 2 hours. *S. aureus* USA300 or kd-SaeR cultures (treated with isoliensinine, 8–64 µg/mL) were grown to OD₆₀₀ nm = 0.5 and added to the plates. After incubation at 37°C, the unbound bacteria were washed away with PBS, and the cells were fixed with 4% paraformaldehyde for 30 minutes. Crystal violet staining was performed, and the absorbance at 595 nm was measured^[35].

2.13 Cell Invasion Assay

ATDC5 cells were seeded at a density of 2×10^5 cells/well in 24-well plates and incubated overnight at 37°C with 5% CO₂. An overnight culture of *S. aureus* USA300 was diluted 1:100 in 1 mL of medium, and

isoliensinine was added at final concentrations of 32 and 64 $\mu\text{g/mL}$. The bacterial suspension was incubated at 37°C and 200 rpm until the OD₆₀₀ reached 1.0. USA300 and kd-SaeR were used as negative and positive controls, respectively. A total of 400 μL of the bacterial suspension was added to the wells, and the plates were incubated in a biochemical incubator at 37°C for 2 hours. The cells were washed three times with PBS, 1 mL of high-glucose DMEM containing 300 $\mu\text{g/mL}$ gentamicin was added, and the mixture was incubated for 1 hour at 37°C. ATDC5 cells cultured on coverslips were lysed with 1% Triton X-100 for 30 minutes, and the lysates were subsequently plated on BHI agar for colony counting^[36].

2.14 Live/Dead Staining and Lactate Dehydrogenase (LDH) Assay

ATDC5 cells were seeded at a density of 2×10^5 cells/well in 24-well plates containing high-glucose DMEM supplemented with 10% FBS and incubated at 37°C with 5% CO₂ for 24 hours. The cells were then incubated with fresh medium containing *S. aureus* and isoliensinine at concentrations of 32 and 64 $\mu\text{g/mL}$ for 5 hours at 37°C. The therapeutic effects of isoliensinine on ATDC5 cells were assessed via a live/dead (green/red) assay kit (Beyotime, China), and fluorescence images were captured via a fluorescence microscope^[37]. To evaluate lactate dehydrogenase (LDH) release, the supernatants were collected after 6 hours of incubation, and LDH levels were measured via an LDH assay kit.

2.15 Neutrophil Chemotaxis Assay

Neutrophils were resuspended in HBSS containing 1% BSA. *S. aureus* USA300 was incubated in the presence of various concentrations of isoliensinine at 37°C with shaking at 220 rpm for 4 hours, after which the supernatants were collected. The bacteria were inactivated by exposure to 60°C for 30 minutes. Neutrophil migration was evaluated via the use of 24-well plates equipped with transwell inserts containing 3.0 μm pores. Heat-inactivated USA300 (3×10^8 cells/insert) was placed in the inserts, while the lower chamber was filled with 0.6 mL of HBSS containing 1% BSA. Neutrophils were exposed to the bacterial supernatants at 37°C for 30 minutes and then centrifuged and resuspended in HBSS with 1% BSA. A total of 1.5×10^4 neutrophils were added to the upper chamber and incubated for 1 hour, and nonmigrating cells were discarded^[38]. After incubation, the neutrophils were stained with Alexa Fluor 647-conjugated Ly6G and washed three times with sterile PBS, and fluorescence was measured at emission wavelengths of 535 nm and 488 nm.

2.16 Fluorescence Quenching Assay

Purified SaeR protein was prepared at a concentration of 500 ng/mL in PBS and incubated with various concentrations of isoliensinine (0–0.36 $\mu\text{g/mL}$) at room temperature for 1 min to allow interaction. Fluorescence spectra were obtained via a fluorescence spectrophotometer (Thermo Scientific, USA). The excitation wavelength was fixed at 280 nm, and emission spectra were recorded between 300 and 400 nm. The slit widths for both excitation and emission were set to 5 nm. The fluorescence intensity at 326 nm was monitored to determine the degree of quenching caused by the binding of the isoliensinine to SaeR^[39]. The binding affinity constant (K_A) was calculated via the Stern–Volmer relationship.

2.17 RNA isolation and quantitative PCR of ATDC5 cells

ATDC5 cells were plated at a density of 1×10^7 cells/well in 6-well dishes containing DMEM supplemented with 10% fetal bovine serum (FBS) and incubated for 24 hours. The cells were subsequently infected with *S. aureus* (1×10^5 CFU/mL) for 1 hour, followed by treatment with 50 μ g/mL gentamicin for an additional hour to eliminate extracellular bacteria. The cells were then washed three times with sterile PBS (pH 7.4), and total RNA was extracted via TRIzol reagent. cDNA synthesis was performed via the PrimeScript RT Kit (TaKaRa, China), and RT-qPCR analysis was conducted via the ABI 7900HT system^[40]. The transcript levels were normalized to those of GAPDH, with the primer sequences detailed in Supplementary Table 3.

2.18 Intracellular bacterial fluorescence staining

S. aureus was incubated with isoliensinine at concentrations of 16 and 64 μ g/mL at 37°C for 24 hours, while the control groups received 0.1% DMSO. The bacterial cells were labeled with 5(6)-carboxyfluorescein N-hydroxysuccinimide ester. ATDC5 cells were seeded at a density of 2×10^5 cells/well in 24-well plates containing DMEM supplemented with 10% fetal bovine serum (FBS) and incubated for 24 hours. The cells were then infected with *S. aureus* at 1×10^5 CFU/mL. After 1 hour of infection, the extracellular bacteria were killed by treating ATDC5 cells with 50 μ g/mL gentamicin for 1 hour. The cells were washed three times with sterile PBS (pH 7.4) and fixed with 4% paraformaldehyde for 15 minutes. Following permeabilization with PBS containing 0.1% Triton X-100 for 10 minutes, the cells were stained with TRITC-phalloidin and DAPI^[41]. Images were captured via a confocal laser scanning microscope (Leica TCS DMI8, Germany).

2.19 In vitro catheter infection model

Sterile polyurethane triple-lumen central venous catheters were incubated with an *S. aureus* suspension ($OD_{600} = 1.0$) at 37°C for 15 minutes. The catheter segments were gently rinsed 2–3 times with sterile PBS and then transferred to 1 mL of fresh rabbit plasma containing fibrinogen (37.5 μ g/mL) and isoliensinine at concentrations of 32 and 64 μ g/mL. The mixture was incubated at 37°C for 24 hours, after which the catheters were rinsed with sterile PBS and fixed with glutaraldehyde (4°C, overnight). Following postfixation with 2% OsO₄ for 2 hours, the samples were dehydrated with ethanol in a graded series for 10 minutes, freeze-dried, and immersed in hexamethyldisilazane overnight^[39]. The catheters were gold coated, and bacterial growth on the surface was observed via a field emission scanning electron microscope (SU8100).

2.20 Cellular Thermal Shift Assay (CETSA)

E. coli BL21 (DE3) harboring pET28a-saeR plasmids was induced to express SaeR protein by the addition of 0.1 mM IPTG. Following sonication, the supernatant was collected by centrifugation. The supernatant was incubated with isoliensinine (32 μ g/mL) at 37°C for 1 hour and then centrifuged at 12000 rpm for 20 minutes at 4°C. The samples were heated at different temperatures for 5 minutes, rapidly cooled in an ice bath for 3 minutes, and centrifuged again at $18,000 \times g$ for 20 minutes at 4°C^[42]. The supernatant was analyzed by SDS-PAGE, and protein intensity was visualized via Coomassie Brilliant Blue G-250 staining. The relative intensity of the proteins was quantified via ImageJ 1.45 software.

2.21 Electrophoretic mobility shift assay (EMSA)

FAM-labeled DNA fragments containing promoter regions were synthesized (as shown in Supplementary Table 3). Binding reactions were performed in buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 5 mM MgCl₂, and 10% glycerol via SaeR protein, DNA probes, and an isoliensinine. The reactions were incubated at 25°C for 30 minutes. The samples were then subjected to electrophoresis on a 5% native polyacrylamide gel^[43]. Fluorescence imaging was performed, and the band intensity was analyzed via ImageJ software.

2.22 Surface plasmon resonance (SPR)

Interaction analysis between the isoliensinine and SaeR was performed via an OpenSPR instrument (Nicoya, Canada). The ligand protein was diluted in binding buffer (10 mM HEPES, 120 mM NaCl, 3 mM EDTA, 0.005% Tween 20, pH 7.4), while the isoliensinine was diluted in PBS containing 1% DMSO. After signal equilibration in PBS with 1% DMSO, imidazole and NiCl₂ solutions were introduced to functionalize the chip surface. The ligand protein was immobilized on the chip, and a 240-second association phase followed by a 240-second dissociation phase was applied^[44]. The surface was exposed to various concentrations of the isoliensinine to determine the maximum binding affinity. The results were analyzed via TraceDrawer software (Ridgeview Instruments AB, Sweden) and a one-to-one binding model.

2.23 Molecular Docking and Site-Directed Mutagenesis

The X-ray crystallographic structure of SaeR (PDB ID: 4QWQ) was retrieved from the Protein Data Bank. The 3D structure of the isoliensinine was constructed to visualize its electrostatic potential via ABEE. Molecular docking of SaeR and the isoliensinine was performed via the standard protocol in AutoDock Tools 1a.5.6, and interactions were analyzed with Discovery Studio. On the basis of the identified binding site, site-directed mutagenesis was conducted via a mutagenesis kit to substitute the corresponding amino acids in SaeR with alanine (mutation primers are listed in Supplementary Table 3). Fluorescence quenching was used to further analyze isoliensinine's inhibition of SaeR mutants^[45].

2.24 Long-term safety assessment

To evaluate the long-term safety of isoliensinine in rats, healthy Sprague–Dawley rats (6–8 weeks old, 8 rats per group) were randomly divided into two groups: a control group and an isoliensinine treatment group (80 mg/kg). This dose was set at twice the highest pharmacologically active dose from the efficacy study to ensure a substantial safety margin. The isoliensinine-treated group received daily subcutaneous injections of isoliensinine (80 mg/kg) for 28 days. The control group received injections of an equal volume of solvent. The animals were observed daily for general appearance, posture, locomotion, fur condition, and food and water intake. Survival was monitored throughout the experimental period. Following the conclusion of the dosing period, the rats were euthanized via inhalation of isoflurane anesthesia. Major organs, including the heart, liver, spleen, lungs, and kidneys, were harvested. After tissue fixation, the samples were embedded in

paraffin. Sections were cut at a thickness of 5 μm and subjected to histopathological examination via H&E staining, followed by observation under an optical microscope.

2.25 *Galleria mellonella* infection model

Galleria mellonella survives at mammalian physiological temperatures of 37°C and possesses an innate immune system similar to that of mammals, including blood cells, melanin deposition, and antimicrobial peptide responses. Its large body size, low cost, and ease of handling make it widely used for studies of *S. aureus* infection and antimicrobial activity evaluation [46, 47]. The efficacy of isoliensinine against *S. aureus* was evaluated via the *Galleria mellonella* infection model. Larvae were divided into five groups: the USA300 infection group, an untreated control group, two isoliensinine treatment groups (20 and 40 mg/kg), and a vancomycin treatment group (20 mg/kg), with each group containing 10 larvae. Infection was induced by injecting 10 μL of an MRSA suspension (5×10^6 CFU/mL) into the proleg on the left side of each larva. One hour post-infection, treatment commenced with the administration of isoliensinine or vancomycin at the designated doses, whereas the control group received an equivalent volume of saline. Larvae were maintained at a constant 37°C, and survival rates were monitored every 12 hours for 96 hours. At 48 hours post-infection, the larvae were euthanized and homogenized, and the bacterial load was quantified by plating the homogenate on BHI agar and incubating it at 37°C for 24 hours. This method established a detection threshold of 100 CFU/mL for the larval homogenate^[48].

2.26 Rat Model of Septic Arthritis

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC). Male Sprague–Dawley rats (6–8 weeks old) were anesthetized with inhaled isoflurane. The hair around the knee joint was shaved, and the area was gently cleansed with warm water. The skin was sterilized via povidone-iodine and alcohol pads.

Septic arthritis was induced by injecting 10 μL of an MRSA suspension (4×10^6 CFU/10 μL) into the intra-articular space below the patella via a 10 μL Hamilton syringe. The treatment groups received subcutaneous injections of vancomycin (20 mg/kg) or isoliensinine (10 mg/kg, 20 mg/kg, 40 mg/kg) once daily for 6 days. The control group received saline injections of the same volume. After infection, the rats were placed on a warming pad and monitored until they regained mobility. All the animals were housed in ventilated cages at 22±3°C, with free access to food and water. Knee joint diameters were recorded, and images of the infected joints were taken daily. On day 7, the rats were euthanized via CO₂ asphyxiation at a controlled low flow rate (30% of the chamber volume per minute)^[49].

Bioluminescence imaging was performed after injecting 10 μL of *S. aureus* USA300 *LAC::lux* (4×10^6 CFU/10 μL) into the joint space below the patella. The average fluorescence intensity of the infected knee joint was monitored in real time on days 1, 2, 3, 4, 5, 6, and 7 post infection. For Safranin O/Fast Green staining, the knees of the rats were harvested on day 7 post euthanasia, fixed in 4% paraformaldehyde, decalcified with 12% EDTA, embedded in paraffin, and sectioned at a thickness of 4 μm . Staining was performed according to the manufacturer's instructions, and stained tissue images were captured via a BioTek

Cytation 5 cell imaging multimode reader. Analysis was conducted via BioTek Gen5 software (BioTek Instruments Inc.).

To quantify the bacterial load, 10 μ L of PBS was injected into the knee joints via a U-100 microfine insulin syringe, aspirated, and transferred to sterile microcentrifuge tubes. The samples were plated on BHI agar supplemented with 10 μ g/mL chloramphenicol and incubated at 37°C for 24–48 hours for colony counting.

To measure the levels of relevant inflammatory factors in rat serum, we collected serum via the abdominal aorta of the rats on the sixth day and detected the relevant levels of TNF- α , IL-1 β , and IL-6 in the rat serum via ELISA kits.

2.27 Micro-CT analysis

Micro-CT analysis of the femoral and tibial condyles was conducted via a NEMO Micro-CT (NMC-200, Pingseng Scientific, China) system. The scanning parameters were set to 80 kV and 0.06 mA, with a scan time of 200 seconds. Three-dimensional reconstructions were performed via Recon software, and data acquisition was completed via Cruiser software (AnalyzeDirect, Inc., USA). The cortical tissue mineral density (TMD), tissue volume (TV), and bone volume (BV) of the condyles were directly measured. The trabecular and cortical BV/TV ratios were calculated^[29].

2.28 Statistical analysis

All the experiments were repeated at least three times to ensure the robustness and reliability of the results. The data are expressed as the means $\pm$ standard deviations. Prior to performing parametric tests, we assessed the necessary assumption: normality was tested by the Shapiro–Wilk test, and homogeneity of variance was tested by Levene’s test. When parametric assumptions were met, differences between two groups were assessed via two-tailed Student’s *t* tests, and multigroup comparisons were performed via one-way ANOVA. Statistical analyses were performed via GraphPad Prism software (version 10). Statistical significance was defined as $*P < 0.05$, with $**P < 0.01$ denoting strong significance and $***P < 0.001$ indicating highly significant differences.

3. Results

3.1 Isolinsinine Targets the SaeR Virulence Regulator in *S. aureus*

The lotus seed embryo (*lian zi xin*), derived from the plumule of *Nelumbo nucifera*, is widely consumed across East and Southeast Asia as both a food ingredient and a traditional medicinal component. As illustrated in Figure 1a, lotus plumule is commonly incorporated into health-promoting food products such as tea, porridge, and snacks. Beyond its dietary applications, recent studies have begun to reveal its potential role in combating pathogenic microorganisms, including drug-resistant bacteria.

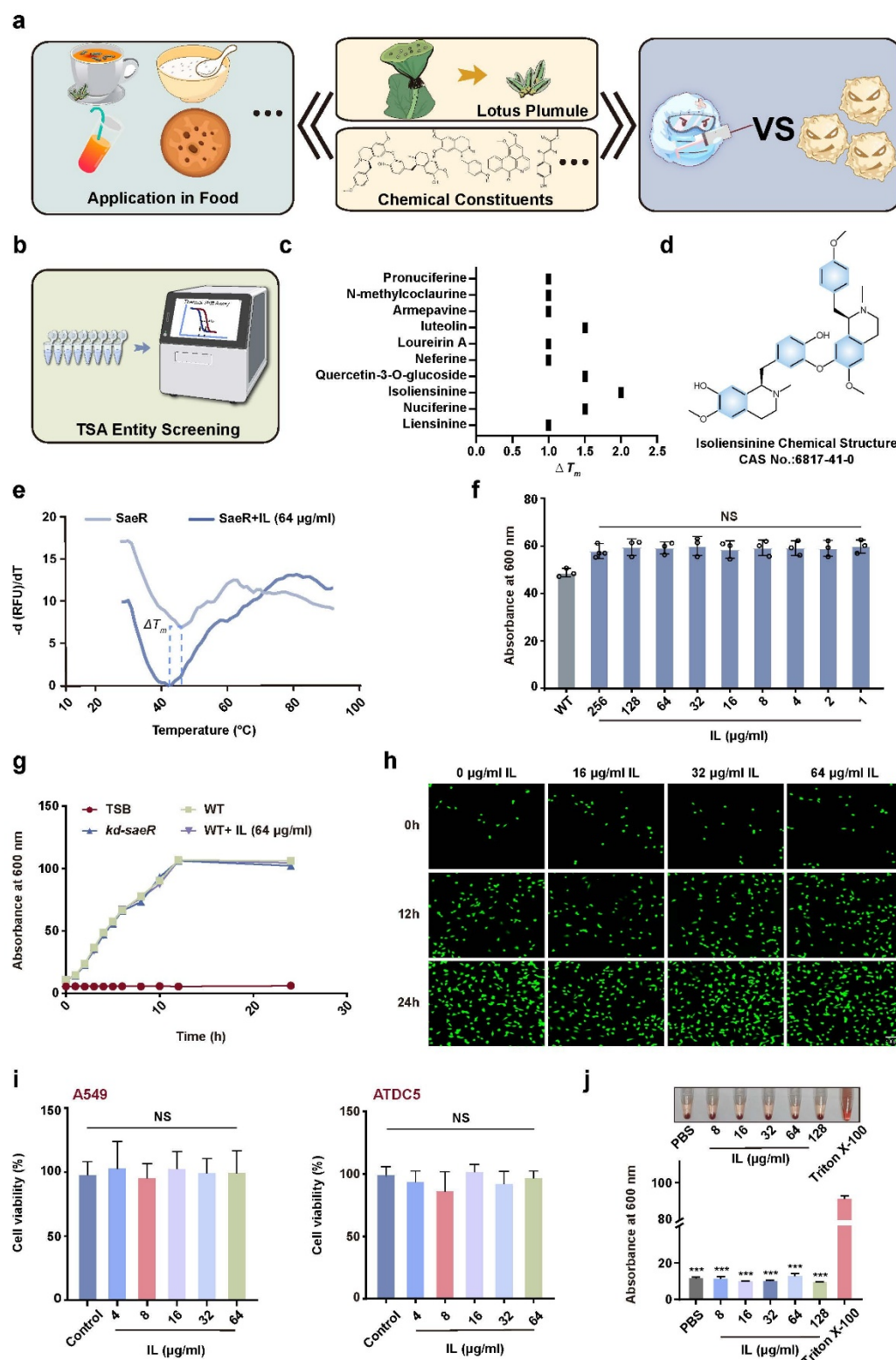


Figure 1. Isolensinine as an inhibitor of *S. aureus* SaeR.

(a) Food applications of lotus plumule and the discovery of its antibacterial potential against *S. aureus*. (b) Schematic of the screening process for SaeR inhibitors from natural small molecules via virtual screening and a thermal shift assay. (c) TSA analysis of major alkaloids screened from lotus plumules. (d) Chemical structure of the isolensinine. (e) TSA analysis of the impact of the isolensinine on SaeR thermal stability. (f) Minimum inhibitory concentration (MIC) of the isolensinine against *Staphylococcus aureus* USA300 (WT group) compared with that of the WT group. (g) Growth curves of *S. aureus* USA300 with and without isolensinine treatment (64 $\mu\text{g/mL}$). (h) Effects of different concentrations (0–64 $\mu\text{g/mL}$) of isolensinine on cell proliferation. (i) MTT assay of the effects of isolensinine at various concentrations (0–64 $\mu\text{g/mL}$) on A549 and ATDC5 cell viability compared with that of the control group. (j) Hemolysis assay of isolensinine at various concentrations in rabbit erythrocytes, with statistical analysis, compared with the Triton X-100 group. Comparative results are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. The error bars indicate the standard error of the mean (SEM).

To investigate this potential, we selected ten major bioactive constituents from lotus plumules and evaluated their ability to interfere with bacterial virulence regulation. Specifically, we examined their interactions with SaeR, a critical response regulator in *Staphylococcus aureus* that governs the expression of multiple virulence factors, via a thermal shift assay (TSA) (Figure 1b). TSA is a widely utilized biophysical technique that detects ligand-induced changes in protein thermal stability, facilitating the identification of compounds that interact with target proteins. Prior to screening, the recombinant SaeR protein was successfully purified via a His-tag affinity system (Supplementary Figure 1). Among the tested compounds, isoliensinine induced a significant positive shift in SaeR melting temperature, indicating a strong stabilizing interaction and suggesting effective binding to the target protein (Figure 1c–e). Based on these results, isoliensinine was selected as the lead candidate for subsequent in vitro and in vivo investigations.

This finding not only reveals a previously unrecognized bioactivity of isoliensinine, a food-derived natural compound, but also underscores the broader potential of functional dietary components in modulating bacterial virulence. Such antivirulence strategies may serve as effective adjuncts to conventional antimicrobial therapies, particularly in the fight against multidrug-resistant pathogens.

3.2 Isoliensinine does not impact the growth of *S. aureus* and exhibits excellent biosafety

Unlike the bactericidal action of antibiotics, antivirulence agents are designed to attenuate the production of virulence factors without directly inhibiting bacterial growth. To investigate whether the isoliensinine affects the growth kinetics of *S. aureus* USA300, we determined its minimum inhibitory concentration (MIC), which was 512 µg/mL, via the microdilution method (Figure. 1f). As shown in Figure 1g, no significant difference in growth rate was observed between the 64 µg/mL isoliensinine -treated group and the DMSO control group, indicating that the isoliensinine does not hinder the growth of *S. aureus* USA300, which is consistent with the expected properties of antivirulence agents.

Furthermore, calcein-AM staining was used to assess the cellular safety of isoliensinine, showing no effect on cell proliferation even at isoliensinine concentrations up to 64 µg/mL (Figure. 1h). Cytotoxicity assays revealed that isoliensinine at concentrations as high as 64 µg/mL had no adverse effects on the viability of A549 or ATDC5 cells, demonstrating its favorable biosafety profile (Figure. 1i). Additionally, the isoliensinine did not exhibit notable hemolytic activity at concentrations up to 128 µg/mL (Figure 1j). A 28-day long-term safety study in rats revealed no animal death at a dose of 80 mg/kg, with all the animals exhibiting normal activity levels (Supplementary Figure 3a). H&E-stained sections of major organs revealed intact cellular structures, with no evidence of inflammatory cell infiltration, necrosis, or degenerative changes (Supplementary Figure 3b). These findings provide a robust foundation for further exploration of isoliensinine as a potential antivirulence agent.

3.3 Isoliensinine suppresses hemolysin activity and virulence factor expression in *S. aureus* In Vitro

Alpha-hemolysin, a critical virulence factor in *S. aureus*, facilitates the disruption of host cell membranes, leading to cell lysis and contributing to the pathogen's invasive capabilities. Given the central role of Hla in *S. aureus* pathogenicity, hemolysis assays showed that isoliensinine effectively inhibited

Hla-induced erythrocyte lysis, achieving an inhibition rate of $34.31 \pm 1.65\%$ at $64 \mu\text{g/mL}$ ($P < 0.001$; Figure 2a). The hemolytic ring test further confirmed that isoliensinine can inhibit the hemolytic activity of *S. aureus* in a dose-dependent manner, thereby reducing damage to red blood cells (Figure 2b).

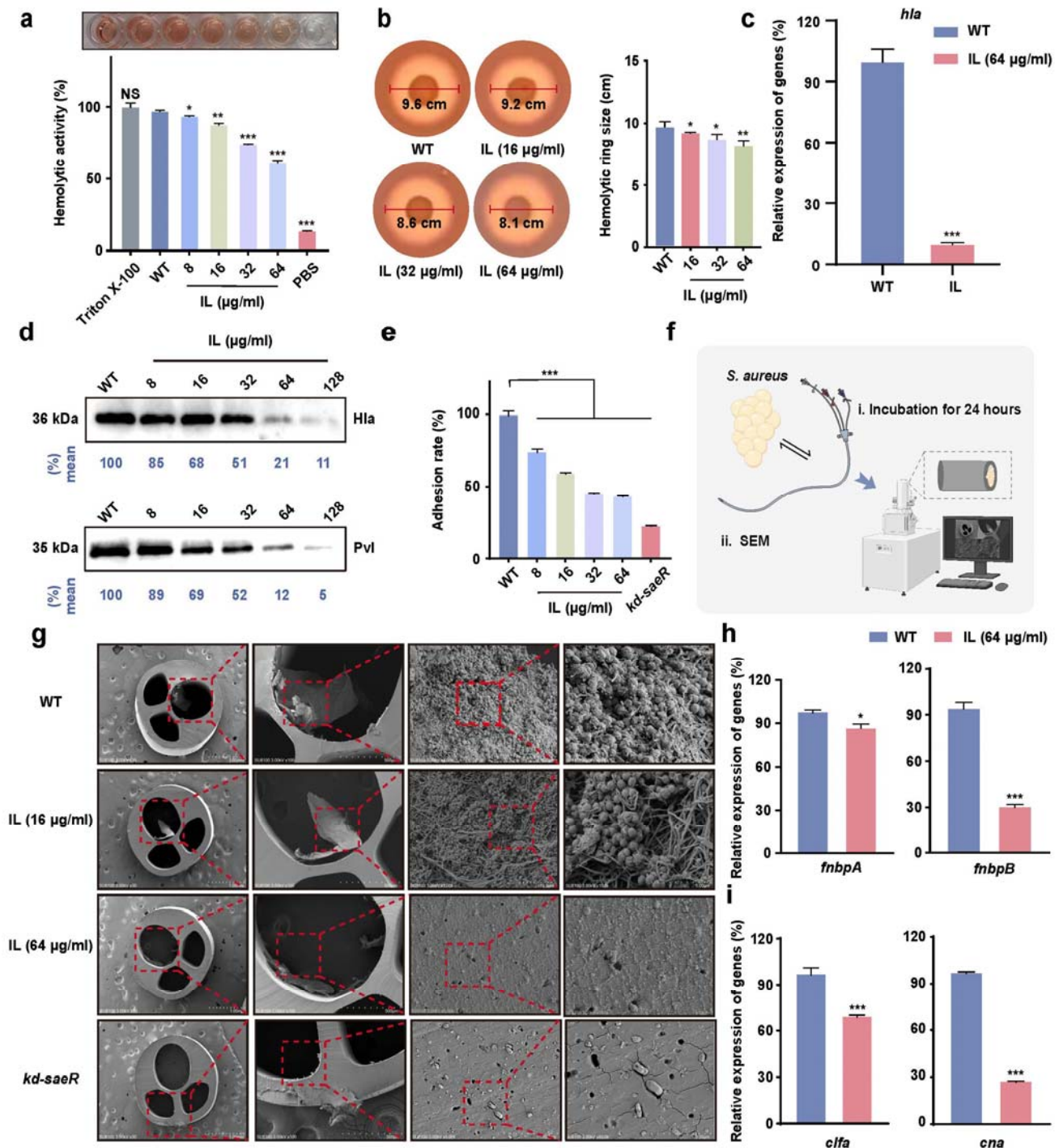


Figure 2. Effect of isoliensinine on SaeR-regulated hemolysis and adhesion-associated virulence factors.

Assessment of the influence of the isoliensinine on hemolytic activity, with observations of dose-dependent reductions in hemolysis proportional to the isoliensinine concentration. (b) Dose-dependent effect of the isoliensinine on Hla activity in *S. aureus*. (c) RT-qPCR analysis of the effect of the isoliensinine on the transcript levels of the *hla* virulence gene. (d) Western blot analysis of the effect of the isoliensinine on Hla and Pvl expression in *S. aureus* USA300, with the molecular weights labeled to the left of each band. (e) Fibrinogen-binding assay evaluating the effects of the isoliensinine on the adhesion of *S. aureus* USA300. (f-g) Representative SEM images of *S. aureus* catheter adhesion with varying isoliensinine concentrations (magnification: 30x, 100x, 3000x, and 10,000x). (h) RT-qPCR analysis of the effect of isoliensinine on the transcript levels of the *fnbpA*, *fnbpB*, *clfa* and *cna* virulence genes. All the statistical comparisons were made against the WT group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The error bars indicate the SEM.

We subsequently examined *hla* expression by qPCR, which showed that isoliensinine (64 µg/mL) reduced *hla* transcript levels by $89.73 \pm 0.25\%$ ($P < 0.001$; Figure 2c). Moreover, we sought to evaluate the effect of isoliensinine on Hla secretion. Western blot analysis showed that isoliensinine markedly reduced Hla secretion, with an $88.81 \pm 0.78\%$ decrease at 64 µg/mL, significant at $P < 0.001$ (Figure 2d and Supplementary Figure 4). In addition to Hla, Pantone-Valentine leukocidin (PVL), a bicomponent leukocidin, is known for its role in immune evasion and tissue destruction during *S. aureus* infection. To explore the broader antivirulence effects of the isoliensinine, we evaluated its impact on PVL expression in *S. aureus* USA300. As shown in Figure 2d and Supplementary Figure 4, isoliensinine significantly inhibited PVL secretion in a concentration-dependent manner, reducing secretion by $94.60 \pm 1.94\%$ at a concentration of 64 µg/mL ($P < 0.001$). These findings suggest that isoliensinine possesses the capacity to modulate multiple virulence pathways in *S. aureus*, further enhancing its therapeutic potential. These results highlight isoliensinine's robust inhibitory activity against key virulence factors in *S. aureus* and pave the way for future investigations into its mechanism of action.

3.4 Isoliensinine inhibits the adherence of *S. aureus*

Given the association of SaeR regulation with bacterial adhesion, we further assessed whether isoliensinine could interfere with *S. aureus* adhesion to fibrinogen. The results indicated that treatment with 64 µg/mL isoliensinine reduced the adhesion rate to 44.18% (Figure. 2e). Further scanning electron microscopy (SEM) studies revealed a dose-dependent effect of isoliensinine on the adhesion of *S. aureus* USA300 to catheters, with 64 µg/mL isoliensinine significantly reducing bacterial adhesion and aggregation on the inner wall of the catheter (Figure. 2f-g). Further quantitative analysis of *saeR*-regulated adhesion-related genes showed that 64 µg/mL isoliensinine significantly inhibited the expression of *fnbpA*, *fnbpB*, *clfa*, and *cna* (Figure. 2h).

3.5 In vitro inhibition of SaeR-associated virulence factor transcription by the isoliensinine in *S. aureus*

RT-qPCR analysis revealed that, compared with the WT, the isoliensinine at a concentration of 64 µg/mL significantly decreased the transcription levels of SaeRS TCS components and SaeR-regulated virulence factors, including *hla*, *pvl*, and other virulence-associated genes, such as *hlgc*, leukocidin E (*lukE*), *splA*, *map*, phenol-soluble modulins α (*psm-α*), *coa* and *vWbp* (Figure. 3a-b). In addition, the transcription level of *chp*, a gene involved in immune evasion, was markedly reduced following isoliensinine treatment (Figure 3a). To further investigate the protective effects of isoliensinine, RNA from ATDC5 cells infected with isoliensinine-treated or untreated *S. aureus* was extracted and subjected to RT-qPCR analysis. The results indicated that isoliensinine treatment led to a significant reduction in the transcription levels of cartilage damage-associated markers, including *adamts5*, *coll0a1*, and *mmp-13* (Figure 3c-e).

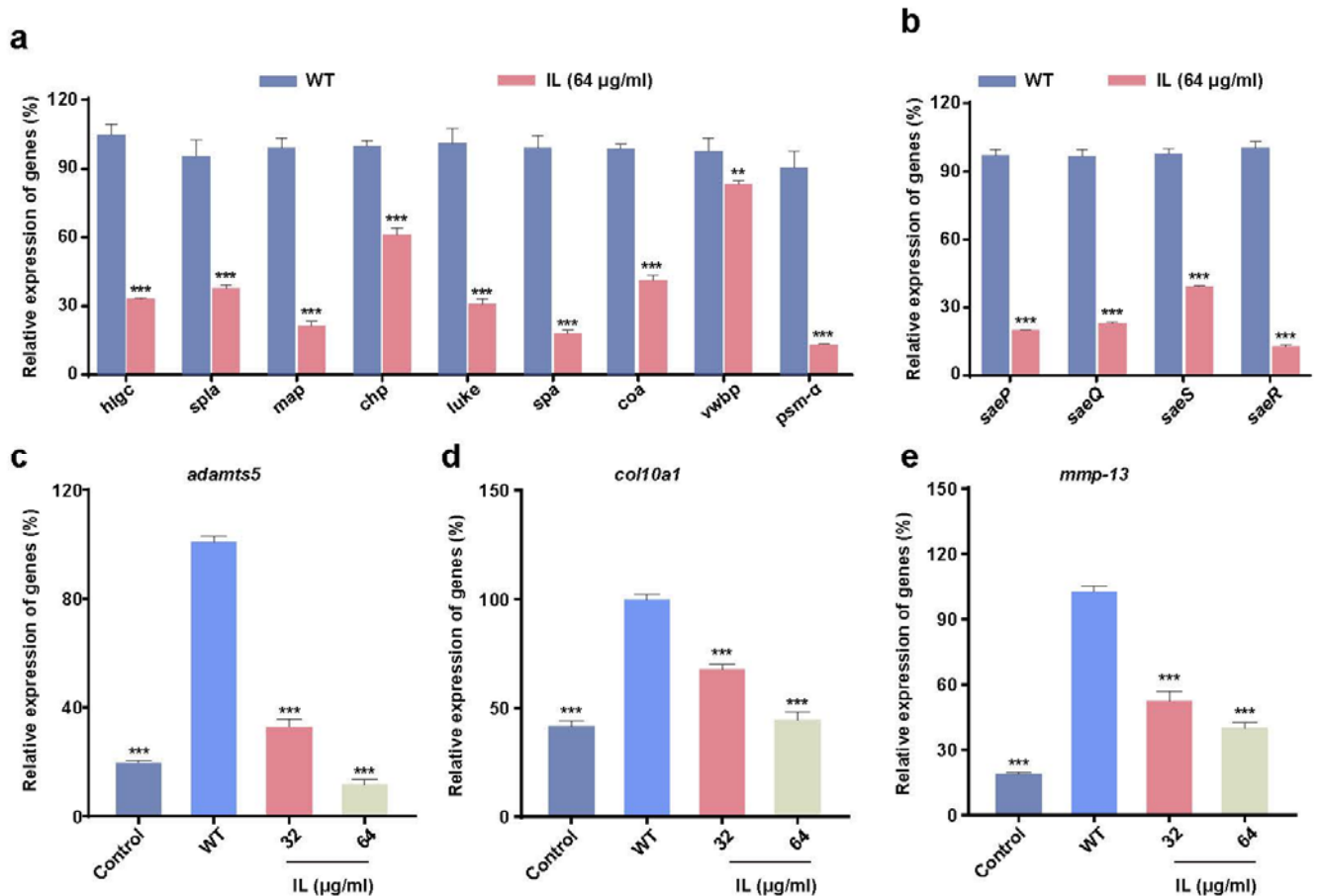


Figure 3. Isoliensinine effects on SaeR-related virulence genes and on bone- and joint-related genes

RT-qPCR analysis of the effect of isoliensinine on the transcript levels of SaeR-regulated virulence genes. (b) RT-qPCR analysis of the effect of the isoliensinine on the transcript levels of SaeR two-component-related genes. (c-e) RT-qPCR analysis of the effects of different concentrations of isoliensinine on *S. aureus*-influenced gene expression in ATDC5 cells. All the statistical comparisons were made against the WT group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The error bars indicate the SEM.

Collectively, these findings indicate that isoliensinine profoundly modulates SaeR-mediated transcriptional regulation in *S. aureus*, leading to alterations in the expression of multiple virulence factors. Therefore, isoliensinine holds promise as an effective tool for treating *S. aureus* infections by altering bacterial virulence characteristics.

3.6 Isoliensinine Reduces *S. aureus* Internalization in ATDC5 Cells and Mitigates ATDC5 Cell Damage

We assessed the ability of *S. aureus* to invade cells with or without isoliensinine treatment. Our results showed that isoliensinine treatment reduced the internalization of *S. aureus* into ATDC5 cells (Figure 4a). Furthermore, the CFU counts of the intracellular bacteria confirmed that 64 µg/ml isoliensinine reduced intracellular bacterial load by 49.8%. (Figure 4b-c).

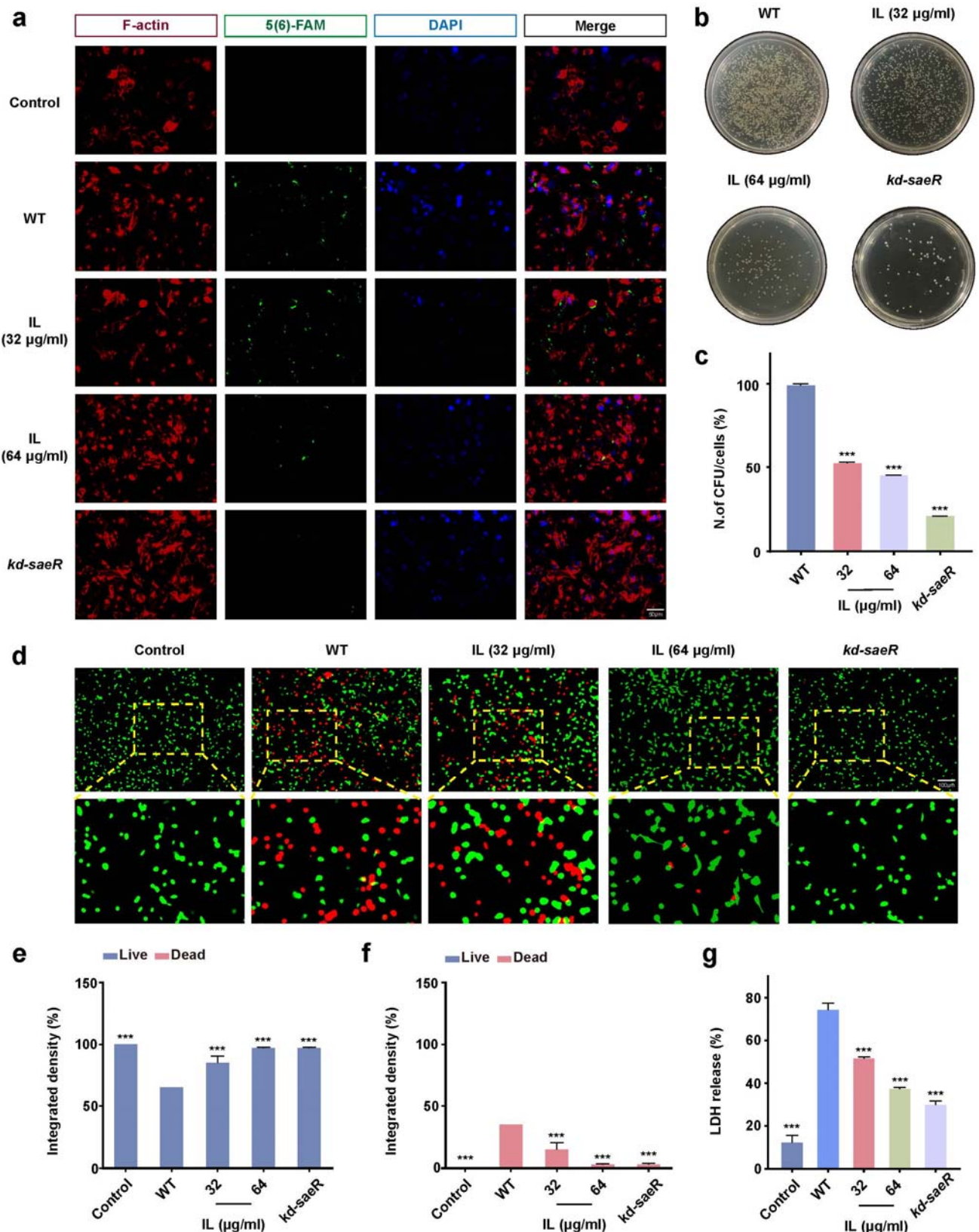


Figure 4. Isoliensinine enhances neutrophil chemotaxis and mitigates *S. aureus*-induced ATDC5 cell damage.

(a) Influence of the isoliensinine on *S. aureus* internalization into ATDC5 cells (scale bar: 50 µm). (b) Results of the inhibitory effect of the isoliensinine on the ability of *S. aureus* to invade ATDC5 cells. (c) Statistical results of the inhibitory effect of isoliensinine on *S. aureus* invasion of ATDC5 competent plate colonies. (d-f) Live/dead staining to evaluate the protective effect of isoliensinine on ATDC5 cells during *S. aureus* infection (scale bar: 100 µm). (g) LDH release analysis in ATDC5 cells. All the statistical comparisons were made against the WT group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The error bars indicate the SEM.

In subsequent experiments, we evaluated the impact of isoliensinine on *S. aureus*-induced damage to ATDC5 cells. Calcein-AM/PI staining was performed to visualize viable and damaged cells, revealing that most ATDC5 cells exposed to USA300 died, as evidenced by elevated LDH levels and an increased proportion of PI-positive cells. In contrast, isoliensinine-treated cells presented significantly reduced LDH release and a lower percentage of PI-positive cells (Figure 4d-g). These findings suggest that isoliensinine effectively protects host cells from USA300-induced detrimental effects, thereby increasing the sensitivity of the host immune response to *S. aureus*.

3.7 Direct interaction between the isoliensinine and *S. aureus* SaeR

To confirm the interaction between the isoliensinine and the SaeR protein, we first conducted CETSA, which demonstrated that the isoliensinine directly binds to the SaeR protein in cells, thereby altering its thermal stability (Figure 5a and supplementary Figure 5).

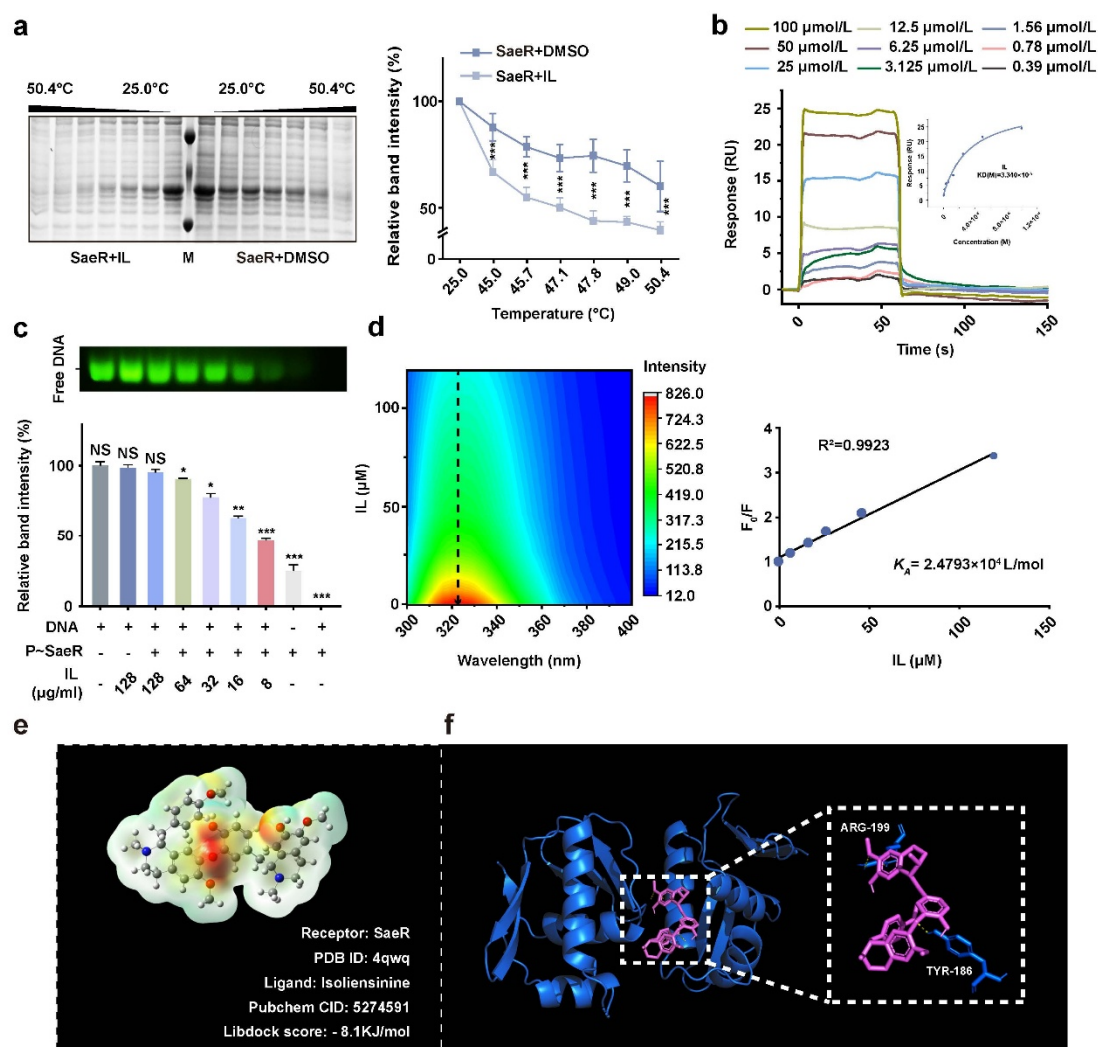


Figure 5. Direct interaction between the isoliensinine and SaeR.

(a) CETSA analysis of the effect of the isoliensinine on SaeR thermal stability. (b) SPR analysis of isoliensinine-SaeR binding kinetics, with color-coded dynamic signal responses. (c) Dose-dependent inhibition of the SaeR-P1 promoter interaction by the isoliensinine, with relative band intensity analysis, compared with the group with an isoliensinine concentration of 128 μg/ml supplemented with the SaeR and P1 promoters. (d) Fluorescence quenching assay setup and affinity results of the isoliensinine with SaeR. (e) ABEE-derived 3D electrostatic potential map of the isoliensinine. (f) Molecular docking illustrating key amino acid interactions between the isoliensinine and SaeR. Comparisons to WT/Control are denoted by * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The error bars indicate the SEM.

Surface plasmon resonance (SPR) was employed to characterize the interaction dynamics between the isoliensinine and SaeR. The calculated dissociation constant (K_D) was 3.34×10^{-5} M (Figure 5b), indicating a strong interaction between the isoliensinine and SaeR. The results of fluorescence quenching provide further evidence of strong binding between the isoliensinine and SaeR proteins (Figure 5d). Further evidence from electrophoretic mobility shift assays (EMSAs) confirmed that the SaeR protein exhibited specific affinity for the P1 promoter fragment, whereas the isoliensinine competitively inhibited the binding of SaeR to the P1 promoter in a dose-dependent manner, validating its regulatory function (Figure 5c).

Subsequently, molecular docking techniques were employed to simulate interactions at the amino acid level and map electrostatic potential distributions on molecular surfaces, thereby determining optimal docking orientations and interaction sites. These simulations revealed that the isoliensinine binds primarily through hydrogen bonds and electrostatic interactions with the SaeR binding pocket. Specifically, residues TYR-186 and ARG-199 were found to have strong hydrogen bonding interactions with the isoliensinine (Figure 5e-f).

To validate these molecular docking results, we performed site-directed mutagenesis on these critical amino acid residues and conducted fluorescence quenching assays. Compared with the wild-type protein, the isoliensinine exhibited reduced inhibitory activity against mutated TYR-186 and ARG-199, confirming the binding of the isoliensinine to SaeR (Supplementary Figures 6-7). As the concentration of the isoliensinine increased, the fluorescence intensity of the SaeR mutants continued to decrease, with the binding affinity constant (K_A) of TYR-186 being the lowest. These findings collectively indicate a strong interaction between the isoliensinine and SaeR.

3.8 Isoliensinine Protects *Galleria mellonella* Larvae from *S. aureus* Infection

Given the accessibility and simplicity of *Galleria mellonella* larvae as model organisms and their innate immune system, which shares substantial similarities with that of mammals, this model has been increasingly adopted for studying microbial infections. To further investigate the systemic protective efficacy of the isoliensinine, we evaluated its ability to protect larvae from *S. aureus* infection at different dosages (Supplementary Figure 9a).

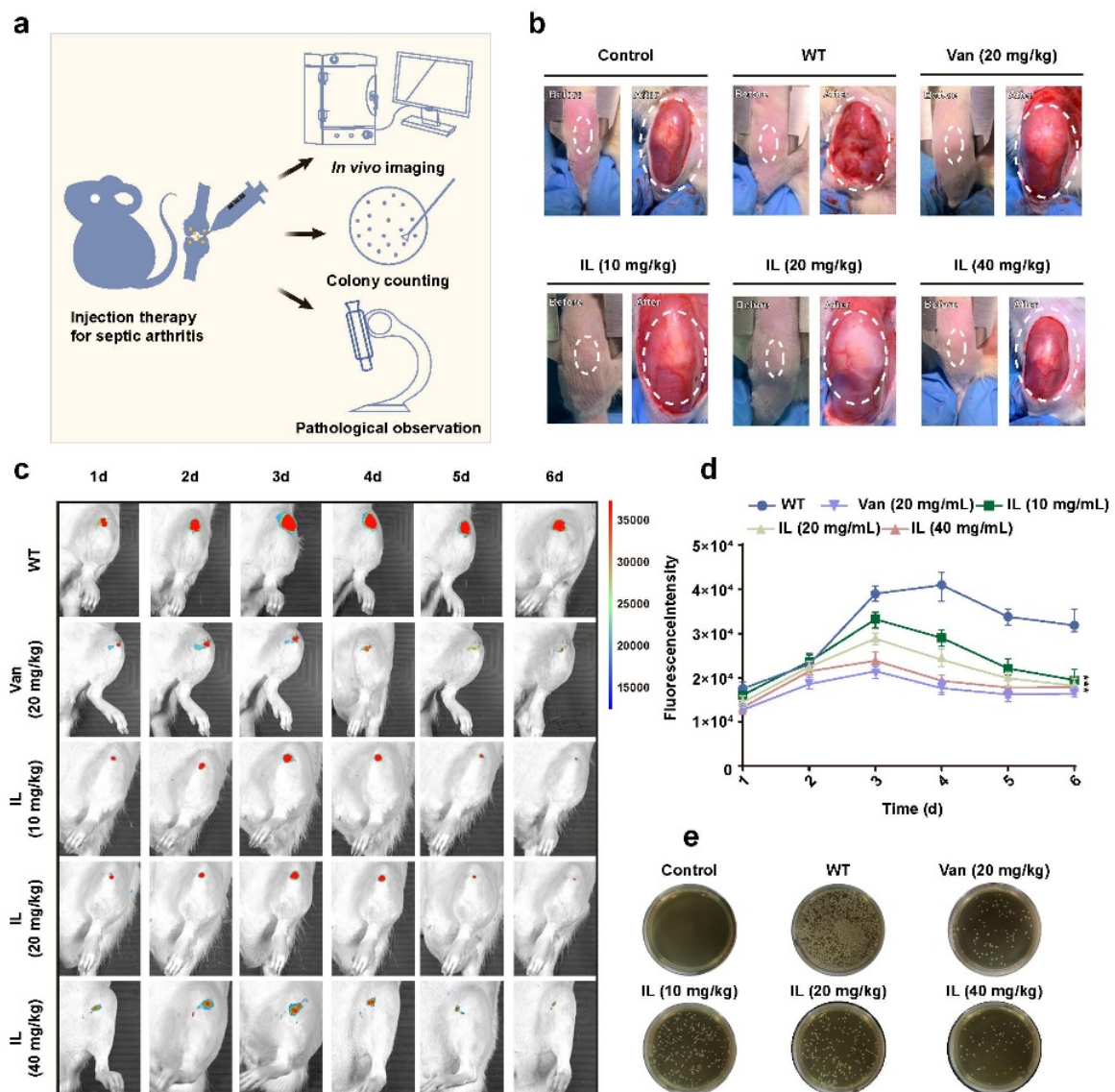
First, to extend our assessment of in vivo toxicity, *Galleria mellonella* larvae were treated with isoliensinine at 20 or 40 mg/kg via injection into the last right proleg. The health status, melanization, and survival of the larvae were monitored daily for 5 days post-treatment. No mortality was observed in the *G. mellonella* larvae, indicating that both 20 and 40 mg/kg isoliensinine were well tolerated (Supplementary Figure 9b-c). The infected larvae experienced 100% mortality within 120 hours without treatment. However, isoliensinine treatment markedly improved survival in a dose-dependent manner. Notably, the survival rate of larvae treated with 40 mg/kg isoliensinine increased to 60% (Supplementary Figure 8 and supplementary Figure 9d).

Bacterial load analysis further substantiated the protective effect of isoliensinine. Sterile larvae were harvested 48 hours post-infection, and colony-forming units (CFUs) were enumerated. Isoliensinine treatment reduced the bacterial burden to 27.2%, a substantial decrease in pathogen load (Supplementary Figure 9e).

These findings underscore the potential of IL as an antivirulence agent, demonstrating not only its safety but also its efficacy in mitigating MRSA-induced immune melanization and mortality in *G. mellonella* larvae, reinforcing its candidacy for further preclinical evaluation.

3.9 Isoliensinine Mitigates Damage in a Rat Model of *S. aureus*-Induced Septic Arthritis

We next sought to further evaluate the therapeutic potential of isoliensinine *in vivo*. A septic arthritis model was established by intra-articular injection of *S. aureus* into SD rats, followed by treatment with isoliensinine at a dose of 10 mg/kg, 20 mg/kg or 40 mg/kg (Figure 6a). Compared with infected control rats, rats treated with vancomycin or isoliensinine also presented significantly alleviated physiological symptoms of septic arthritis, such as knee joint swelling (Figure 6b). Fluorescence imaging of the knee joints was performed daily throughout the observation period. Notably, both the vancomycin and isoliensinine treatment groups presented a lower mean bioluminescence intensity (indicative of reduced infection severity) after reaching peak values than did the untreated WT strain infection group (Figure 6c-d).



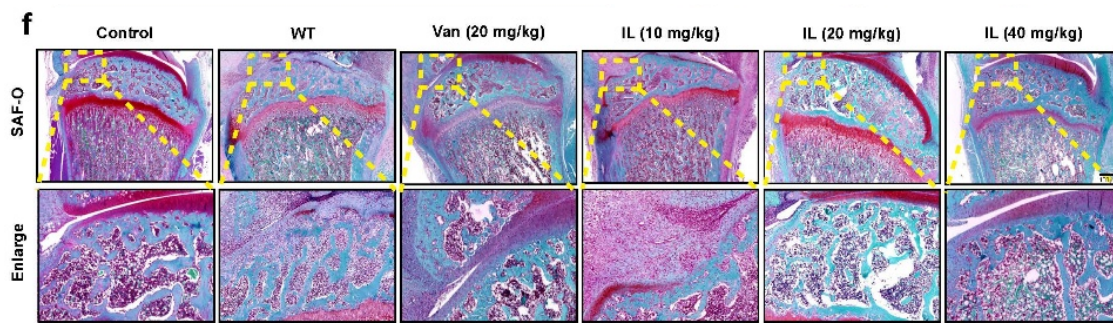


Figure 6 Effects of isoliensinine on septic arthritis induced by *S. aureus* infection.

(a) Schematic of the *S. aureus*-induced septic arthritis rat model and subsequent treatment protocol. (b) Representative pre- and posttreatment knee joint images. (c-d) Representative bioluminescence images and intensity quantification over days 1–7 in the septic arthritis model (n=5), with the color bar indicating the relative photon intensity (p/s/cm²/sr) compared with that of the WT group. (e) Results of colony counting of knee rinses. (f) Safranin O/Fast Green staining of paraffin-embedded tissue. Comparative results are indicated by * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The error bars indicate the SEM.

To assess the histological changes in the knee joints and bacterial burden among the different groups, we performed colony counts on knee tissue rinses and observed a significant reduction in the number of colonies in the isoliensinine-treated group (Figure 6e and supplementary Figure 10). In addition, knee joint tissues were collected on day 7 postinfection and subjected to Safranin O/Fast Green staining. Compared with the WT group, vancomycin and isoliensinine treatment improved MRSA-induced cartilage degradation, bone erosion, and bone formation (Figure. 6f), highlighting isoliensinine's ability to mitigate joint pathological damage in bacterial synovial infections.

Micro-CT was used to visualize the bony structure of the knee joints directly under infection conditions. Three-dimensional (3D) images revealed significant erosion of the cortical and trabecular bone in the distal femur and proximal tibia of MRSA-infected rats (Figure 7a). MRSA infection led to reduced bone volume/total volume (BV/TV) and tissue mineral density (TMD) of both cortical and trabecular bone in the femur and tibia. Treatment with vancomycin or isoliensinine alone significantly improved the MRSA-induced BV/TV changes (Figure 7b). Further testing of inflammatory factor levels revealed that treatment with vancomycin or isoliensinine alone significantly reduced the levels of TNF- α , IL-1 β , and IL-6 (Figure 7c-e).

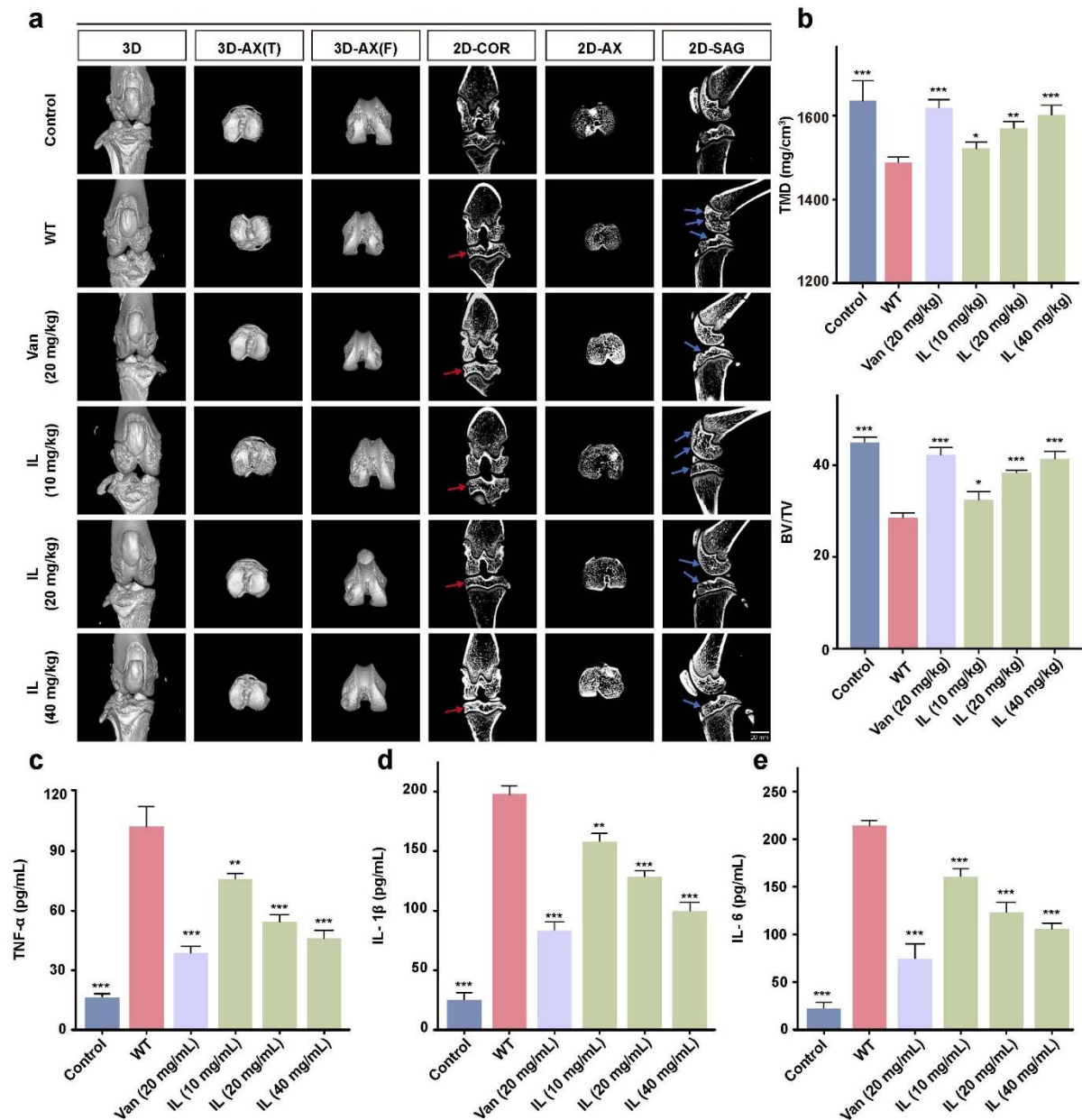


Figure 7 Micro-CT image showing septic arthritis caused by isoliensinine-mediated attenuation of *S. aureus* infection.

(a) 3D and 2D micro-CT reconstructions of the femur and tibial condyle surfaces in knee joint specimens. Red arrows indicate areas of cortical bone destruction, trabecular bone rarefaction, and bone volume loss. The blue arrows point to regions of articular cartilage defects or thinning. (b) Quantitative analysis of BV/TV and TMD via micro-CT analysis ($n=3$). (c-e) Serum levels of the inflammatory factors TNF- α , IL-1 β , and IL-6 in the rats in each group. All the statistical comparisons were made against the WT group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The error bars indicate the SEM.

4. Discussion

Staphylococcus aureus is a leading cause of persistent and relapsing bacterial infections, particularly those associated with inflammatory damage and immune evasion. With the increasing prevalence of antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA), conventional antibiotic therapies are often rendered less effective and may contribute to the emergence of resistance. As a result, alternative strategies that do not rely solely on bacterial eradication are gaining traction. Among these, antivirulence approaches—which aim to suppress the expression or function of pathogenic factors without directly affecting

bacterial viability—offer the dual advantage of reducing disease severity while minimizing selective pressure for resistance development [21].

In recent years, naturally occurring compounds from edible and medicinal plants have emerged as promising candidates for antivirulence therapy. These bioactive molecules, which are commonly found in functional foods, have the potential to modulate pathogen behavior and support host defense. The lotus seed embryo (*lian zi xin*), a widely consumed food and traditional health supplement in East Asia and Southeast Asia, contains several alkaloids with known antioxidative and anti-inflammatory activities. In this study, we identified isoliensinine, a bisbenzylisoquinoline alkaloid derived from lotus seed embryos, as a potent inhibitor of *S. aureus* SaeR—a key virulence regulator—highlighting a novel functional role for this food-derived compound in mitigating bacterial pathogenicity.

Infection by *S. aureus* is initiated through a complex adhesion process that facilitates stable colonization within host tissues.^[18, 50] *S. aureus* expresses multiple adhesion-related virulence factors that facilitate its attachment to host cells and extracellular matrix components, creating a foundation for deeper tissue invasion. Key adhesion proteins, including clumping factor A (*clfA*), fibronectin-binding proteins A and B (*fnbpA* and *fnbpB*), and collagen-binding protein (*cna*), mediate interactions with host proteins such as fibronectin, fibrinogen, and collagen, enhancing bacterial attachment and colonization in the joint environment^[18-20, 51]. These interactions are particularly critical in septic arthritis, as the successful adhesion of *S. aureus* to host tissues is a prerequisite for biofilm formation, immune evasion, and subsequent tissue invasion. Our findings revealed that isoliensinine significantly downregulates the expression of these adhesion-related virulence genes, including *clfA*, *fnbpA*, *fnbpB*, and *cna*, which are essential for establishing infection. This suppression of adhesion factors suggests that isoliensinine reduces the pathogen's ability to attach to the joint surfaces, thereby impeding the initial steps of infection. Furthermore, recent studies have demonstrated that the depletion of von Willebrand factor-binding protein (vWbp) significantly diminishes *S. aureus*'s ability to invade joint cavities while having minimal impact on other organs, suggesting a mechanism specific to joint tissues^[52]. In our experiments, we observed a reduction in vwbp expression, which was consistent with this joint-specific pattern. Both in vitro catheter adhesion assays and cartilage cell internalization assays further confirmed that isoliensinine effectively reduces the ability of MRSA to adhere to and invade chondrocytes. These findings suggest that isoliensinine disrupts critical pathways for joint-specific invasion, positioning it as a promising therapeutic candidate for mitigating MRSA-related septic arthritis.

Research has shown that Hla plays a dominant role in chondrocyte damage and death during septic arthritis caused by *S. aureus* infection, highlighting potential therapeutic targets for cartilage protection^[53]. Hla binds to ADAM10 receptors on chondrocytes, leading to pore formation in the cell membrane and subsequent influx of Na⁺ and water, which results in cell lysis. This interaction underscores Hla's critical role in cartilage destruction^[54, 55]. Our findings demonstrate that isoliensinine significantly reduces Hla secretion, thereby attenuating the virulence of *S. aureus*. In addition to its effects on Hla, the isoliensinine also modulates other virulence factors regulated by the SaeR/S two-component system (TCS), which orchestrates

the expression of various toxins and immune evasion proteins, such as *pvl*, *hlgC*, and *lukE*. By downregulating these factors, isoliensinine diminishes *S. aureus* virulence, thereby minimizing damage to chondrocytes in MRSA-infected cartilage, as confirmed by live/dead cell assays that underscore its protective effects.

Our findings reveal that isoliensinine not only downregulates immune evasion-related genes, including *pvl* and *chip*, which are essential for *S. aureus* evasion of host defenses but also enhances neutrophil chemotaxis. This dual action promotes neutrophil recruitment to the infection site while simultaneously weakening bacterial evasion mechanisms. These effects include reduced bacterial virulence and improved host immune defense. They together indicate the therapeutic potential of isoliensinine against *S. aureus* infection.

The *Galleria mellonella* infection model provides a practical *in vivo* platform for the early evaluation of isoliensinine, as its innate immune system shares key features with those of mammals and can be studied at 37°C [56, 57]. In our experiments, isoliensinine improved larval survival in a dose-dependent manner and reduced the bacterial burden. The extent of bacterial load reduction differed from that observed in rat joint infection models, potentially due to the simpler immune system of the *Galleria mellonella* and differences in tissue permeability. In summary, these findings confirm its protective role in simple and cost-effective systems.

Mechanistically, the action of the isoliensinine is grounded in its binding affinity for SaeR, as confirmed by CETSA and SPR, which demonstrated stable interactions and disruption of the regulatory function of SaeR [58]. Molecular docking and mutagenesis studies identified Tyr-186 and Arg-199 as crucial residues for isoliensinine binding, further supporting the specificity of the inhibitory action of the isoliensinine on SaeR.

Isoleinsinine, a bisbenzylisoquinoline alkaloid from lotus seed embryos, acts through a highly specific nonbactericidal mechanism by directly binding the *S. aureus* regulator SaeR, thereby suppressing adhesion factors, hemolysins and immune evasion proteins, which collectively reduce pathogenicity, enhance neutrophil chemotaxis and protect tissues during infection-induced inflammation. Unlike isoliensinine, traditional plant derived antimicrobials often depend on broad and nonspecific bactericidal actions. Epigallocatechin, the major component of green tea extract, represents this conventional mode by exerting antimicrobial activity against *S. aureus* [59] through membrane disruption, biofilm inhibition and interference with virulence related pathways [60–65], effects that can impose selective pressure and increase the likelihood of resistance. Additionally, dose conversion based on the BSA method indicates that effective isoliensinine exposure cannot be achieved through lotus seed consumption alone, as it would require approximately 132 to 789 seeds per day [66, 67], highlighting concentrated extracts as the only practical route for application. Building on this mechanism and dose feasibility, isoliensinine shows promise as a virulence attenuating ingredient for incorporation into food matrices such as meat or dairy products and for development as a nutritional supplement or functional food for high risk populations. Overall, this study highlights the potential of food-derived bioactive compounds in modern anti-infective research and underscores the broader application of dietary interventions in promoting microbial homeostasis and long-term health.

Data availability statements

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest

The authors declare that they have no conflicts of interest related to this work.

Ethics statement

The animal work in this report was approved by the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences, and it complied with the regulations on the use of experimental animals.

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