Supplementary Material B – Extended Methods for:

Limosilactobacillus reuteri-derived tripeptide SKL exerts antibacterial activity against systemic and wound infections via dual membrane-killing and anti-virulence mechanisms

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Bacterial strains and culture conditions

Salmonella Typhimurium SL1344, *Salmonella* Typhimurium SSH006, *Salmonella* Pullorum MY185, *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* CMCC46117, *Enterobacter cloacae* SH0421, *Pseudomonas aeruginosa* MJF02-2, *Acinetobacter baumannii* B27981, *Enterobacter hormaechei* IF3516, *Staphylococcus aureus* CMCC26003, methicillin-resistant *Staphylococcus aureus* N315 (MRSA), *Listeria monocytogenes* L0019, *Enterococcus faecalis* CQ24TZQY02703, *Bacillus cereus* IF3306, *Lacticaseibacillus rhamnosus* P118, and *Lactiplantibacillus plantarum* 16 were kindly provided by the laboratory of Prof. Min Yue (Hangzhou Institute for Advanced Study, University of Chinese Academy of Sciences, Hangzhou, Zhejiang, China). Two additional *Limosilactobacillus reuteri* strains, P190 and P126, were isolated in our laboratory from the intestinal contents of healthy one-month-old piglets.

All strains were maintained at -80°C in 20% glycerol and propagated under species-specific conditions. Lactic acid bacteria were cultured anaerobically at 37°C in de Man, Rogosa, and Sharpe (MRS) broth or on MRS agar. Gram-negative pathogens (e.g., *S.* Typhimurium SL1344) were grown aerobically at 37°C in Luria-Bertani (LB) medium with shaking (180 rpm), whereas Gram-positive pathogens (e.g., *S. aureus* N315) were grown aerobically at 37°C in Brain Heart Infusion (BHI) medium. To minimize phenotypic variation, all strains were passaged no more than three times prior to use.

Isolation and identification of lactic acid bacteria from piglet intestinal contents

Intestinal contents were collected from healthy one-month-old piglets under sterile conditions and divided into two aliquots for parallel isolation of lactic acid bacteria (LAB) and non-LAB probiotics. For LAB isolation, 0.1 g of intestinal sample was suspended in 9.9 mL MRS broth and incubated anaerobically at 37°C overnight. After serial 10-fold dilution in 0.9% NaCl, 100 μL aliquots of appropriate dilutions were plated on MRS agar and incubated anaerobically at 37°C for 24 h. Milky-white single colonies were picked and purified by two successive streaks on MRS agar. For

non-LAB probiotic isolation, the same amount of intestinal content was incubated aerobically overnight at 37°C in shaking conditions, followed by identical dilution and plating on BHI agar with aerobic incubation at 37°C for 24 h. Three colonies with different morphologies were selected and purified by two additional streaks on BHI agar.

All purified isolates were individually inoculated into 1 mL MRS (LAB, anaerobic) or BHI (non-LAB, aerobic with shaking) broth for 24 h, then mixed with 40% glycerol (1:1) and stored at -80°C. Bacteria were identified by MALDI-TOF MS (Bruker Daltonics) per the manufacturer's instructions.

Screening of antibacterial activity by agar well diffusion assay

Antibacterial activity of *L. reuteri* isolates was assessed using the agar well diffusion method with Oxford cups. Briefly, Oxford cups (8 mm diameter) were placed in empty Petri dishes, which was then filled with molten LB agar. After solidification, the Oxford cups were aseptically removed with forceps, generating uniform wells. Indicator bacteria were grown overnight, diluted to ~ 1×10^8 CFU/mL in fresh LB broth, and 200 µL of this suspension was spread evenly over the agar surface. Each well received 200 µL of test sample, such as *L. reuteri* culture, cell-free supernatant, or other solutions. Following incubation at 37°C for 16-18 h, the diameters of inhibition zones were measured and recorded.

Untargeted metabolomics analysis of Cell-free supernatants from *L. reuteri* P190

Cell-free supernatants from *L. reuteri* P190 (high activity) and P126 (weak activity) were subjected to untargeted metabolomics analysis. Following methanol precipitation, samples were analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) at Novogene Technology Company (Beijing, China). Metabolite identification and relative quantification were performed using Compound Discoverer software (Thermo Fisher Scientific). Differentially

accumulated metabolites between the two strains were defined as a $\log_2$(fold change) > 1 and $p < 0.05$.

Growth inhibition assay

S. Typhimurium SL1344 was grown overnight, then diluted 1:100 in fresh LB broth and incubated until mid-log phase ($OD_{600} \approx 0.5$). Bacterial suspension was then diluted to approximately 5×10^5 CFU/mL. In a 96-well plate, 100 µL of diluted bacterial suspension was mixed with 100 µL of test samples (undiluted or diluted) and incubated at 37°C with shaking. Bacterial growth was tracked by measuring OD_{600} every hour over 24 h using a microplate reader (BioTek). All assays were performed in triplicate.

Minimum inhibitory concentration (MIC) determination

The peptides SKL, its tandem-repeat variants, and APK were chemically synthesized (Sangon Biotech) and stored as lyophilized powders at -20°C. Immediately before each assay, the peptides were dissolved in sterile deionized water to prepare stock solutions (up to 5 mg/mL) and used without delay. MICs were determined by the broth microdilution method. Briefly, bacterial strains were grown to mid-log phase and diluted to ~5×10^5 CFU/mL in Mueller-Hinton broth (MHB). Two-fold serial dilutions of each peptide in MHB were mixed with equal volumes of bacterial suspension in 96-well plates. After incubation at 37°C for 16 h, the MIC was recorded as the lowest peptide concentration that prevented visible bacterial growth.

Hemolysis assay

Hemolytic activity of the peptides was assessed using mouse erythrocytes. Blood was collected from healthy six-week-old female C57BL/6 mice by orbital sinus puncture under isoflurane anesthesia. Erythrocytes were isolated by centrifugation (1,000 × g, 10 min, 4°C), washed three times with sterile PBS, and resuspended in PBS to a final concentration of 4% (v/v). Serial dilutions of each peptide were mixed with an equal volume of erythrocyte suspension in 96-well plates. PBS and 0.1%

Triton X-100 served as negative and positive controls, respectively. After incubation at 37°C for 1 h with gentle shaking, the plates were centrifuged at 1,000 × g for 10 min. Supernatant (100 μL) was transferred to a new plate, and absorbance at 540 nm was measured. Hemolysis percentage was calculated as: Hemolysis (%) = [(A_sample - A_PBS) / (A_Triton - A_PBS)] × 100.

Mammalian cell viability assay

The cytotoxicity of the peptides was assessed using the murine macrophage cell line RAW264.7. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO_2. Cells were seeded into 96-well plates at 1 × 10^4 cells per well and cultured overnight. Peptides were added at a concentration equivalent to twice the highest MIC value recorded for each peptide against any bacterial strain tested, and incubation continued for 24 h. Cell viability was determined using the Cell Counting Kit-8 (CCK-8; APExBIO) according to the manufacturer's instructions. Briefly, 10 μL of CCK-8 solution was added to each well, followed by a further 2 h incubation at 37°C. Absorbance at 450 nm was measured with a microplate reader. Cell viability was calculated as: Cell viability (%) = [(As - Ab) / (Ac - Ab)] × 100, where As represents the absorbance of peptide-treated wells, Ab is the absorbance of blank wells, and Ac is the absorbance of untreated wells. Viability was expressed relative to untreated control cells.

Biofilm inhibition assay

The effect of peptides on biofilm formation by *S.* Typhimurium SL1344 was evaluated using a crystal violet staining assay. Overnight bacterial cultures were diluted to ~ 1 × 10^6 CFU/mL in LB broth, and 100 μL aliquots were dispensed into 96-well plates. Peptides (SKL or $(SKL)_4$) were added at final concentrations of ¼×, ½× and 1× the MIC. Polymyxin B at 4×MIC served as a positive control, and untreated wells served as negative controls. Plates were incubated statically at 37°C for 24 h. After incubation, planktonic cells were removed, and wells were gently washed twice with PBS. Adherent biofilms were fixed with methanol for 15 min,

air-dried at 25°C, and stained with 0.1% crystal violet (Solarbio) for 15 min. Excess stain was removed by washing with water, and the plates were air-dried. The bound crystal violet was solubilized in 95% ethanol, and biofilm mass was quantified by measuring absorbance at 590 nm using a microplate reader.

Antibiotic resistance development assay

To evaluate the propensity for resistance development, *S.* Typhimurium SL1344 and methicillin-resistant *Staphylococcus aureus* (MRSA) were serially passaged in subinhibitory concentrations of test compounds. Bacteria were cultured in CAMHB containing 0.5×MIC of SKL, $(SKL)_4$, polymyxin B (for *S.* Typhimurium SL1344), or vancomycin (for MRSA). After 24 h of incubation at 37°C, cultures were diluted 1:100 into fresh medium containing the same antibiotic or peptide concentrations. This process was repeated for 20 consecutive cycles. The fold changes in MIC at each passage were calculated relative to the starting MIC (passage 0).

Scanning electron microscopy (SEM)

To assess peptide-induced morphological alterations, *Salmonella* Typhimurium SL1344 and MRSA were examined by scanning electron microscopy. Bacteria were grown to mid-log phase, harvested and resuspended in PBS at ~1 × 10^5 CFU/mL. Suspensions were then incubated with SKL or $(SKL)_4$ at 1×MIC for 2 h at 37°C. Untreated cells incubated in PBS serve as controls. Following incubation, cells were collected and fixed overnight at 4°C in 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2). The fixed cells were washed three times with PBS and dehydrated in a graded ethanol series (30%-100%, 10 min per step). Samples were then critical-point dried, mounted onto aluminum stubs, and sputter-coated with gold. Imaging was performed using a Nova NanoSEM 650 scanning electron microscope (FEI) at an accelerating voltage of 3 kV.

Membrane permeability assays

Membrane integrity was assessed using two complementary fluorescence- based

assays. Outer membrane permeability was evaluated via the N-phenyl-1-naphthylamine (NPN) uptake assay. *S.* Typhimurium SL1344 was collected at mid-log phase, washed, and resuspended in 5 mM HEPES, supplemented with 5 mM glucose (pH 7.2) to an OD_{600} of 0.5. NPN was added to a final concentration of 20 μM, and the bacterial suspension was distributed into black 96-well plates. Following the addition of SKL or $(SKL)_4$ at 1×MIC, fluorescence was measured immediately (excitation at 350 nm, emission at 420 nm) using a fluorescence microplate reader (BioTek), with readings taken every hour for 6 h.

Inner membrane permeability was assessed by measuring the hydrolysis of o-nitrophenyl-β-D-galactopyranoside (ONPG). *S.* Typhimurium SL1344 was collected at mid-log phase, washed, and resuspended in PBS containing 1.5 mM ONPG to an OD_{600} of 0.5. After adding peptides at 1×MIC, absorbance at 420 nm was measured at 20-min intervals over 120 min using a microplate reader.

Membrane fluidity assay

Membrane fluidity was evaluated by measuring the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated into bacterial membranes. *Salmonella* Typhimurium SL1344 was collected at mid-log phase, washed three times with PBS, and resuspended in PBS to an OD_{600} of 0.5. The bacterial suspensions were then incubated with SKL or $(SKL)_4$ at 1×MIC for 2 h at 37°C. Untreated cells incubated with PBS serve as controls. After treatment, cells were collected, and DPH was added to a final concentration of 2 μM. The mixture was incubated in the dark at 37°C for 45 min. Excess DPH was removed by centrifugation, and the cells were washed twice with PBS and resuspended in saline. Fluorescence polarization was measured using a Hitachi F-7000 spectrophotometer (excitation at 360 nm and emission at 430 nm). The fluorescence polarization value (P) was calculated as: $P = (I_{vv} - I_{vh}) / (I_{vv} + I_{vh})$, where I_{vv} represents the fluorescence intensity with both polarizers oriented vertically, and I_{vh} represents the intensity with the polarizer vertical and analyzer horizontal.

Membrane hydrophobicity assay

Bacterial cell surface hydrophobicity was evaluated using the microbial adhesion to hydrocarbons (MATH).[1] *Salmonella* Typhimurium SL1344 was collected at mid-log phase, washed three times with PBS, and resuspended in PBS to an OD_{600} of 0.5 (designated A_0). The bacterial suspension was then incubated with SKL or $(SKL)_4$ at 1×MIC for 2 h at 37°C. Untreated cells incubated with PBS serve as controls. Following treatment, 2 mL of the suspension was mixed with 1 mL of n-hexadecane, vigorously vortexed, and incubated at room temperature for 30 min to allow complete phase separation. The aqueous phase was carefully withdrawn, and its absorbance at 600 nm was measured (A_1). The percentage of cell surface hydrophobicity was calculated as: Hydrophobicity (%) = (1- A_1 / A_0) × 100%, where A_0 represents the initial absorbance before n-hexadecane addition, and A_1 represents the absorbance of the aqueous phase after n-hexadecane treatment.

Preparation of SKL, $(SKL)_4$ and mupirocin ointments

Ointments were formulated according to a previously described method with minor modifications.[2] Briefly, polyethylene glycol 2000 (PEG2000), polyethylene glycol 200 (PEG200), and glycerin were mixed thoroughly in a glass vial at a weight ratio of 1:1:4. The mixture was then heated to 50 °C in a water bath under constant stirring for 0.5 h to ensure complete homogeneity. Subsequently, SKL, $(SKL)_4$, or mupirocin powder was added to achieve final concentration of 2%, 1%, and 1% (w/w), respectively. After an additional 0.5 h of heating and stirring, the formulation was allowed to cool naturally to room temperature, yielding a pale yellow semi-solid formulation. A vehicle control ointment was prepared identically without the addition of any antimicrobial agent.

Mouse husbandry and experimental design

All animal experiments were performed using five-week-old specific-pathogen-free (SPF) female C57BL/6J or ten-week-old SPF female BALB/c mice purchased from the SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Mice were maintained

under controlled conditions (25 °C, 12-h light/dark cycle) with free access to sterile water and autoclaved chow. Mice were group-housed at a density of five per cage to ensure social housing and minimize stress. All animal experiments were performed in accordance with the guidelines and protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang University (Protocol No. 38046).

For all measured endpoints, the individual mouse served as the independent experimental unit. Inclusion criteria were predefined prior to the experiments: only healthy, weight-matched SPF mice were enrolled, and no animals or raw data points were excluded from any analysis. Eligible mice were stratified by body weight and randomly assigned to treatment or vehicle control groups using a random number generator. Cage positions and sample testing orders were systematically rotated to reduce confounding effects arising from location and measurement sequence. The researchers responsible for group allocation, injections, and daily monitoring were aware of group identities; however, during for colony-forming unit (CFU) enumeration, wound area measurement, histological scoring, and all statistical analyses, they were fully blinded, using coded sample IDs with no treatment labels. Group sample sizes (n = 5, 6, or 10, as explicitly indicated in all figure legends) followed widely accepted standards for murine infection models, and no formal a priori power analysis was conducted. All experiments were independently repeated at least three times. Mice were monitored daily for body weight and clinical symptoms with standardized humane endpoints to minimize suffering. Isoflurane inhalation anesthesia was employed for full-thickness wound surgery and peripheral blood collection by orbital sinus puncture, and all animals were euthanized at experimental terminal time points in accordance with institutional standardized procedures.

Systemic Salmonella infection model with L. reuteri P190 and cell-free supernatant

To assess *L. reuteri* P190-mediated protection, C57BL/6 mice were randomly assigned to treatment groups (n = 10 per group for survival and body weight

monitoring; n = 6 per group for bacterial load determination). Mice received daily oral gavage of PBS-washed L. reuteri P190 (~1 × 10^9 CFU in 200 µL PBS), filter‑sterilized (0.22 µm) supernatant, trypsin-digested supernatant, or vehicle (sterile PBS) for 7 consecutive days before oral infection with 1 × 10^8 CFU of *S.* Typhimurium SL1344 in 200 µL PBS. Gavage treatment continued after infection. Body weight and survival were monitored daily for up to 20 days. At day 3 post-infection (dpi), mice were euthanized, and liver, spleen, cecal contents, and blood were harvested aseptically. Tissues were homogenized in sterile PBS, serially diluted, and plated on LB agar for colony-forming unit (CFU) enumeration.

Prophylactic and therapeutic efficacy of SKL and $(SKL)_4$ in systemic Salmonella infection

To evaluate the protection efficacy of SKL and $(SKL)_4$, peptides were dissolved in sterile PBS and administered either intraperitoneally (i.p.) or intragastrically (i.g.) in a volume of 100 µL. For dose optimization, C57BL/6 mice received SKL (i.p. or i.g.) or $(SKL)_4$ (i.p. only) at 5–50 mg/kg at 2 h, 24 h, 48 h, and 72 h post-infection following oral infection with *S.* Typhimurium SL1344. Vehicle control received PBS. At 72 h post-infection (dpi 3), 2 hours after the final treatment dose, tissues were collected for bacterial load determination (n = 6 per group). For prophylactic efficacy, mice received daily i.p. injections of 25 mg/kg peptide from day –7 to day –1 before infection, followed by the aforementioned infection and monitoring protocol.

Cyclophosphamide-induced neutropenic mouse model

To evaluate the therapeutic efficacy of SKL and $(SKL)_4$ in immunocompromised mice, neutropenia was induced in C57BL/6 mice by intraperitoneal injection of cyclophosphamide (80 mg/kg) 4 and 2 days before *S.* Typhimurium SL1344 infection. Then the mice were treated with SKL or $(SKL)_4$ (25 mg/kg i.p.) at 2 h, 24 h, and 48 h post-infection. At 48 h post-infection (dpi 2), 2 hours after the final treatment dose, livers, spleens, cecal contents, and blood were collected for bacterial load

determination (n = 6 per group). Vehicle mice received PBS. Body weight was monitored daily, and survival was recorded for 14 days (n = 10 per group).

Fecal microbiota transplantation experiment

Donor C57BL/6J (B6J) mice received daily intraperitoneal injections of SKL (25 mg/kg) or PBS for 7 days. Fecal pellets were collected daily from each donor group, pooled, and suspended in PBS at a concentration of 100 mg/mL. Recipient B6J mice were depleted of their endogenous gut microbiota by administration of an antibiotic cocktail (ampicillin 1 g/L, metronidazole 1 g/L, neomycin 1 g/L, vancomycin 0.5 g/L) in drinking water for 2 weeks. Immediately thereafter, recipients were colonized by daily oral gavage with 200 μL of the corresponding donor fecal suspension for 4 consecutive weeks to ensure stable engraftment. Following the FMT period, recipient mice were orally infected with *S.* Typhimurium SL1344 (1×10^8 CFU). At day 3 post-infection (dpi), bacterial loads in liver, spleen, cecal contents, and blood were quantified (n = 6 per group), and body weight was monitored daily from infection until sampling.

Competitive infection assay

C57BL/6 mice were orally co-infected with a 1:1 mixture of wild-type (streptomycin-resistant) and *ΔqseC* (streptomycin- and spectinomycin-resistant) *S.* Typhimurium strains at a total dose of 1×10^8 CFU per mouse. At 2, 24, and 48 hpi, the mice received intraperitoneal injections of either a subinhibitory dose of SKL (5 mg/kg in 100 μL PBS) or an equal volume of PBS (vehicle control). At 72 hpi, mice were euthanized 2 h after the final treatment (n = 6 per group). Liver, spleen, ileum, cecal contents, and feces were aseptically collected. Tissues were homogenized in PBS, serially diluted, and plated on LB agar containing streptomycin or streptomycin + spectinomycin. The competitive index (CI) was calculated as the output ratio (*ΔqseC*/ wild-type CFU) divided by the input ratio.

316 ***Full-thickness MRSA wound infection model***

317 BALB/c mice were anesthetized with isoflurane, and the dorsal hair was shaved. A
318 full-thickness excisional wound (6 mm diameter) was created on the dorsum using a
319 sterile biopsy punch, followed by inoculation with 20 μL of MRSA suspension (~1 ×
320 10^7 CFU). Two hours after infection, mice received topical application of vehicle
321 ointment, SKL ointment (2%, w/w), $(SKL)_4$ ointment (1 %, w/w), or mupirocin ointment
322 (1%, w/w) every other day. Wound closure was monitored by photographing the
323 wounds on days 3, 5, 7, 9, and 11 post-infection. Wound areas were quantified using
324 ImageJ software**.** At day 6 post-infection, mice were euthanized (n = 6 per group), and
325 wound tissues were excised, homogenized in PBS, serially diluted, and plated on
326 mannitol salt agar for MRSA CFU counting.

327

328 **Histological analysis**

329 Liver and spleen tissues were collected and fixed in 4% paraformaldehyde
330 (Sinopharm Chemical Reagent Co.), dehydrated, and embedded in paraffin. Sections
331 (4 μm thick) were mounted onto glass slides and stained with hematoxylin and eosin.
332 After dehydration, sections were mounted with neutral balsam and imaged under a
333 light microscope (NIKON).

334

335 **Flow cytometry analysis**

336 To verify successful induction of neutropenia, peripheral blood was collected from
337 cyclophosphamide-treated mice prior to *Salmonella* infection. Red blood cells were
338 lysed with RED BLOOD CELL LYSING BUFFER (Sigma), and leukocytes were
339 resuspended in PBS supplemented with 1% FBS. Cells were stained with antibodies
340 targeting CD45 (104), CD3 (145-2C1), CD8a (53-6.7), B220 (RA3-6B2), CD11b
341 (M1/70), and Ly6G (1A8). Dead cells were excluded using 7‑AAD. Antibodies were
342 purchased from Thermo Fisher Scientific or Biolegend. Data acquisition was
343 performed on a NovoCyte™ flow cytometer (Agilent), and analysis was conducted
344 using FlowJo software (BD Life Sciences).

345

Generation of *qseC* knockout and complemented *Salmonella*

To delete *qseC* in *Salmonella* Typhimurium SL1344, we employed a CRISPR-Cas9-assisted λ Red recombination system using the pTarget/pCas dual-plasmid system as previously described.[3] First, the pCas helper plasmid (encoding λ Red recombinases and Cas9) was introduced into SL1344, and recombinase expression was induced with 10 mM arabinose to prepare electrocompetent cells. For the construction of the editing plasmid, a 20−bp single−guide RNA (sgRNA) targeting the *qseC* coding sequence and a donor template comprising two ~500−bp homology arms flanking the *qseC* gene were cloned into the pTarget vector (spectinomycin resistance) to generate the recombinant plasmid pTargetT-*ΔqseC*. This plasmid was then transformed into the pCas−containing competent cells. Recombinants were selected on LB agar supplemented with 100 μg/mL spectinomycin (for pTargetT-*ΔqseC* maintenance) and 50 μg/mL kanamycin (for pCas maintenance). The *qseC* deletion was confirmed by colony PCR and DNA sequencing.

For genetic complementation, the *qseC* gene, including its native promoter region was amplified from SL1344 genomic DNA and cloned into the low-copy vector pACYC184. The resulting recombinant plasmid was electroporated into the *ΔqseC* mutant to generate the complemented strain (C-*ΔqseC*). Transformants were selected on 25 μg/mL of chloramphenicol. Plasmid maintenance and restored *qseC* expression were confirmed by PCR and Western blot analysis, respectively.

RNA extraction and RT-qPCR for bacterial genes

Total bacterial RNA was extracted using the Bacteria RNA Extraction Kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. RNA concentration and purity were assessed by spectrophotometry (Thermo Fisher, Waltham, MA, USA). cDNA was synthesized from 2 μg of total RNA using the HiScript® III RT SuperMix for qPCR (+ gDNA wiper) (Vazyme). Quantitative real-time PCR (qRT-PCR) was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme) on a LightCycler® 480 II real-time PCR system (Roche Diagnostics).

Primer sequences for target genes (*qseC, phoP, lrp, cpxA, hilD, sipA, ssaV, sifA, flhDC, fliC*) and the reference gene (*16S rRNA*) are listed in Supplementary Table 3. All reactions were performed in triplicate. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Bacterial protein extraction and Western blot analysis

Bacterial cells were harvested, washed twice with PBS, and lysed in lysis buffer composed of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2% (w/v) SDS, and a protease inhibitor cocktail (Beyotime). To solubilize the hydrophobic inner membrane protein QseC, the lysates were vortexed vigorously for 30 s, subjected to brief sonication pulses (3–5 × 10 s on ice), and then incubated at room temperature for 10 min. After centrifugation to remove insoluble debris, the protein concentration in the supernatant was measured using a BCA protein assay kit (Beyotime).

Proteins were resolved by 12% SDS-PAGE and electrotransferred onto PVDF membranes (Millipore) by electroblotting. Membranes were blocked with 5% bovine serum albumin (BSA) in TBST (Tris-buffered saline containing 0.1% Tween-20) for 1 h at room temperature, then incubated overnight at 4°C with primary antibodies: mouse polyclonal anti-QseC antibody (custom-made) and rabbit polyclonal anti-RpoB antibody (Biorbyt). Following three washes with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit secondary antibodies (FD Bio) for 1 h at room temperature. Protein bands were detected using the SuperSignal West Atto Ultimate Sensitivity Substrate (ThermoFisher Scientific) and visualized with a ChampChemi imaging system (SINSAGE). Band intensities were quantified with ImageJ software (NIH), and QseC protein levels were normalized to RpoB.

Statistical analysis

All experiments were repeated at least three times independently under similar conditions. Data are presented as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 9 software. An unpaired two-tailed

Student's *t*-test was applied for two-group comparisons. For multiple-group comparisons, one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used. For two-factor experimental designs, two-way ANOVA with Tukey's post hoc test was employed. Survival curves were analyzed using the log-rank (Mantel-Cox) test. Statistical significance was defined as $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$); "ns" indicates not significant.

References

1. Rosenberg M, Gutnick D, Rosenberg E. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiology Letters 1980; 9:29-33.
2. Zhang S, Zhu Y, Zou J, Zhang M, Li X, Wang Y, et al. Screening of an antibacterial peptide with high antibacterial activity against superbugs and its application in the treatment of skin wound infections. Chemical Engineering Journal 2025; 522:167748.
3. Jiang Y, Chen B, Duan C, Sun B, Yang J, Yang S. Multigene editing in the Escherichia coli genome via the CRISPR-Cas9 system. Applied and environmental microbiology 2015; 81:2506-14.