

Bacillus Subtilis Revives Conventional Antibiotics Against Staphylococcus Aureus Osteomyelitis

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Abstract

As treatment of *Staphylococcus aureus* osteomyelitis is often hindered by the development of antibiotic tolerance, novel antibacterial therapeutics are required. Here we found that the cell-free supernatant of *Bacillus subtilis* (*B. subtilis* CFS) killed planktonic and biofilm *S. aureus*, and increased *S. aureus* susceptibility to penicillin and gentamicin as well. Further study showed that *B. subtilis* CFS suppressed the expression of the genes involved in adhesive molecules (Cna and ClfA), virulence factor Hla, quorum sensing (*argA*, *argB* and *RNAIII*) and biofilm formation (*Ica* and *sarA*) in *S. aureus*. Additionally, our data showed that *B. subtilis* CFS changed the membrane components and increased membrane permeabilization of *S. aureus*. Finally, we demonstrated that *B. subtilis* CFS increased considerably the susceptibility of *S. aureus* to penicillin and effectively reduced *S. aureus* burdens in a mouse model of implant-associated osteomyelitis. These findings support that *B. subtilis* CFS may be a potential resistance-modifying agent against *S. aureus* osteomyelitis.

Introduction

Gram-positive *Staphylococcus aureus* (*S. aureus*) has been identified as the most common causative pathogen for osteomyelitis and other various musculoskeletal infections [1, 2]. *S. aureus* osteomyelitis remains a significant healthcare problem in China and around the world due to high rates of recurrence and treatment failure [3, 4]. Treatment of *S. aureus* infection in bone is complicated by its vast immune evasion, persistence mechanisms and intrinsic antibiotic resistance mechanism. *S. aureus* may secrete multiple virulence factors including immunomodulatory proteins, toxins and superantigens, leading to death of innate immune cells and disturbance of complement activation [5]. As the infection persists and becomes chronic, *S. aureus* may adhere to implanted devices, lacunae-canaliculi in cortical bone or sequestra, thereby forming biofilm phenotype [6, 7]. Once a biofilm forms, *S. aureus* is 10 – 1,000 times more resistant to antimicrobial agents than planktonic bacteria [8] and induces phagocytosis dysfunction of macrophages [9]. Additionally, intracellular persistence of *S. aureus* in osteoblasts, macrophages, osteoclasts or osteocytes may induce immune cell evasion and antibiotic tolerance of *S. aureus* during infection [10, 11]. Furthermore, *S. aureus* has such intrinsic mechanism for antibiotic resistance as decreasing permeability of outer membrane, activating drug efflux systems, and producing excessive β -Lactamase [12–14].

Surgical debridement of necrotic bone combined with long-term administration of antibiotics is a traditional therapy to treat chronic osteomyelitis [15]. Several antibiotics are used for management of *S. aureus* osteomyelitis, such as vancomycin, tobramycin, daptomycin and clindamycin, but the rapid acquisition of resistance to antibiotics by *S. aureus* is a significant problem [16–19]. Therefore, it is urgent to find a more effective antibacterial strategy to prevent occurrence and recurrence of bone infections.

Recently, probiotics such as *Bacillus subtilis* (*B. subtilis*) has been used to prevent infection, because it is a nonpathogenic Gram-positive bacterium which can effectively maintain a beneficial microflora balance

in the gastrointestinal tract of a mammalian host [20]. Accumulating evidence from animal and *in vitro* studies suggests that *B. subtilis* produces various substances, such as surfactins, iturins and fengycins, which may benefit anti-bacterial, anti-inflammatory and immunomodulatory applications [21, 22]. Specifically, a recent report showed that the secreted substance from *B. subtilis* abolished colonization with *S. aureus* by suppressing production of the Arg-quorum-sensing signaling system [21]. In light of recent evidence implicating anti-infection and decolonization role of *Bacillus* lipopeptides against *S. aureus*, we investigated the effect of *B. subtilis* cell-free supernatant (*B. subtilis* CFS) on the growth of *S. aureus in vitro* and *in vivo*.

Here we found that *B. subtilis* CFS exerted a potent antimicrobial function against *S. aureus* and increased its susceptibility to antibiotics as well *in vitro* and *in vivo* as well. Furthermore, we demonstrated that *B. subtilis* CFS changed the membrane components and increased membrane permeabilization of *S. aureus*, which may be associated with increased susceptibility of *S. aureus* to antibiotics. Our data may suggest a new treatment paradigm with existing drugs that have already been approved against infection.

Materials And Methods

2.1 Bacterial strains and culture

S. aureus strains were isolated from the osteomyelitis subjects from Department of Orthopedics, Nanfang Hospital, Southern Medical University, using PHOENIX 100 (Becton Dickinson Microbiology System, USA). *B. subtilis* (CMCC-B-63501) was obtained from China General Microbiological Culture Collection Center. Bacterial strains were cultured in TSB (Cat. LA0110, Solarbio, Beijing, China) at 37 °C under shaking at 200 rpm. Overnight bacterial cultures were collected by a centrifuge, and pellets washed and resuspended in PBS (Cat. C10010500BT, GIBCO, Beijing, China). The bacterial suspensions were adjusted to an OD₆₀₀ of 0.5 measured using a microplate spectrophotometer (CLARIOstar, BMG LABTECH, Germany), approximately equal to 1×10^8 colony forming unit per ml (CFU/ml).

2.2 Preparation of cell-free supernatant from *B. subtilis* culture and treatments

To prepare *B. subtilis* CFS, *B. subtilis* strains were cultured at 37 °C under shaking at 200 rpm overnight until the cultures reached an OD of 0.4 ± 0.05 at 600 nm. The CFS of bacterial culture was collected by centrifugation at 6000 g for 10 min, and then filtered through a 0.22 µm sterilizing-grade filter (Millipore, SLGV033RB, USA) to remove bacteria. The CFS was aliquoted and stored at -20 °C until the day of experimentation.

To evaluate the effect of *B. subtilis* CFS on *S. aureus* genes expression, overnight culture of *S. aureus* strains was collected by a centrifuge, washed with PBS, re-suspended at 1×10^8 CFU/ml in TSB/PBS (1:1

v/v, control) or TSB/*B. subtilis* CFS (1:1 v/v) and incubated in 6-well-plate at 37 °C for 3 hrs. Finally, bacteria were collected for RNA extraction and analysis of genes expression.

2.3 Planktonic bacterial growth assay

To determine the antibacterial effect of *B. subtilis* CFS on *S. aureus*, the growth of planktonic *S. aureus* was assessed using the method as described previously [23] with some modifications. Briefly, 100 µL of *S. aureus* suspension (5×10^8 CFU/mL) from a fresh overnight culture was inoculated into 5 mL TBS/PBS (1:1 v/v, control) or TSB/*B. subtilis* CFS (1:1 v/v), and incubated with shaking at 200 rpm at 37 °C. The growth of *S. aureus* was determined by monitoring OD₆₀₀ of the cell culture at 2, 4, 6, 8, 10, 12 and 24 h after seeding.

2.4 Biofilm formation and viability assay of biofilm *S. aureus*

To evaluate the effect of *B. subtilis* CFS on *S. aureus* biofilm formation, 100 µL of *S. aureus* (5×10^8 CFU/mL) was added to 900 µL of TSB/PBS (1:1 v/v), TSB/*B. subtilis* CFS (1:1 v/v), TSB/PBS (1:1 v/v) with 32 µg/mL penicillin, or TSB/PBS (1:1 v/v) with 0.75 µg/mL in each well on a 12-well plate and incubated at 37 °C for indicated time points without shaking. Next, after the medium removed, the wells were washed three times with sterile PBS. Finally, the plates were air-dried for 45 min and the adherent cells and matrix were stained with 0.1% crystal violet solution.

SYTO9 (Cat. S34854, Invitrogen, Thermo Fisher Scientific) and PI (Cat. P346, DOJINDO, Japan) staining was performed to evaluate the effect of *B. subtilis* CFS on the viability of biofilm *S. aureus*. 100 µL of *S. aureus* (5×10^8 CFU/mL) was added to 900 µL of TSB in each well on a 12-well plate. After 24 h of static incubation at 37 °C, the wells were washed three times with PBS to remove nonadherent cells and refilled with 1 mL/well of the four different sterile culture media: TSB/PBS (1:1 v/v, control), TSB/*B. subtilis* CFS (1:1 v/v), TSB/PBS (1:1 v/v) with 32 µg/mL penicillin, and TSB/PBS (1:1 v/v) with 0.75 µg/mL gentamicin. After 8 h incubation and washing for three times, the biofilm *S. aureus* were stained with 3 µM of PI and 10 µM of SYTO9 in 1 × PBS for 20 min in the dark, and visualized under a fluorescence microscope. Live and dead bacteria were stained green, and dead ones red.

2.5 MIC and killing assay

The potential of synergy was evaluated via MIC evaluation and time-killing assays. MIC was determined using Epsilometer testing (E-test) following the method previously described [24, 25]. Briefly, fresh overnight culture of *S. aureus* was collected and washed twice with PBS, and suspended and pretreated in 1 ml PBS (control) or *B. subtilis* CFS at 1×10^8 CFU/ml for 1 h. Add 150 µl pretreated *S. aureus* suspension was added and spread evenly on a Mueller-Hinton Agar plate. The plate was allowed to dry for 10–15 min before applying E-test strip immobilized with predefined continuous and stable gradients of penicillin (Cat. 921021, Liofilchem, Italy) or gentamicin (Cat. 920090, Liofilchem, Italy). The plates were incubated at 35 °C for 24 h and the MIC value was read at the point where the ellipse intersects the E-test strip.

To monitor the response of *B. subtilis* CFS-pretreated *S. aureus* to penicillin or gentamicin, bacterial growth was continuously monitored over a time-course of 24 h (0, 2, 4, 6, 8, 10, 12, 14, 24 h). 500 μ l of *S. aureus* suspension (1×10^8 CFU/ml) pre-treated with PBS (control) or *B. subtilis* CFS was inoculated into 4.5 mL of Mueller-Hinton broth with penicillin or gentamicin at 0.5 MIC. A 200 μ L of sample was removed from each tube at indicated time points for measuring OD₆₀₀.

For time-killing assay, 500 μ l of *S. aureus* suspension (1×10^7 CFU/ml) pretreated with PBS (control) or *B. subtilis* CFS was inoculated into 4.5 mL of Mueller-Hinton broth with penicillin or gentamicin, with each drug tested at 2 \times MIC and 4 \times MIC. A 10 μ L of sample was removed from each tube at 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 h for colony count enumeration. 10 μ L samples with 100-fold dilutions were plated onto Mueller-Hinton Agar plates and incubated at 35 °C for 18 h. Colonies were counted and the mean CFU/mL from triplicate samples was evaluated.

2.6 RNA extraction and Quantitative real-time PCR (qRT-PCR)

Total RNA of *S. aureus* was extracted with a Bacterial RNA Extraction Kit (B518655-0050, Sangon Biotech, Shanghai, China) following the manufacturer's instructions. RNA purity was checked using a NanoDrop spectrophotometer (ND-1000, Nanodrop, USA). RNA was reversely transcribed using the 5 \times PrimeScript RT Master Mix (RR036A, Takara, Shiga, Japan) according to the manufacturer's instructions. qRT-PCR was performed using TB Green Premix Ex Taq II (RR820A, Takara, Shiga, Japan). The primers sequences are listed in Table 1. Fold changes in expression of each gene versus control were determined using $2^{-\Delta\Delta C_t}$ method with *gyrB* as a housekeeping gene.

2.7 Transmission electron microscopy (TEM)

S. aureus suspension (1×10^8 CFU) pretreated with PBS (control) or *B. subtilis* CFS was collected and fixed in 2.5% Glutaric dialdehyde at 4 °C overnight. After washing, *S. aureus* pellets were dehydrated in a series of ethanol concentrations (50–100%) followed by 100% acetone. Samples were then embedded in Spurr resin (EM0300, Sigma-Aldrich, USA). 50 nm ultrasections were cut using an ultramicrotome (EM UC7, Leica, Germany) and stained with uranyl acetate for 10 min. After being washed with ddH₂O, sections were stained with Reynolds lead citrate for 30 min. Finally, sections were observed on a transmission electron microscope (H-7500, Hitachi, Japan) equipped with a 16 million pixels format CCD camera and images were made at 120 kV in high contrast mode.

2.8 Bacterial membrane permeabilization assays

Fresh overnight culture of *S. aureus* (1×10^8 CFU/ml) was treated with PBS or *B. subtilis* CFS for 1 h, then ATP release assay and SYTO9/PI staining were performed to evaluate the changes in membrane permeability of *S. aureus*. SYTO9/PI staining was performed according to the method described in 5.4. For ATP release assay, the total and extracellular ATP concentrations were detected using BacTiter-Glo™ Microbial Cell Viability Assay Kit (G8230, Promega, USA) and ATP Bioluminescent Assay Kit (FLAA-1KT, Sigma-Aldrich, USA), respectively, according to manufacturing instructions. The amount of light produced

from samples was measured with the integration time of 6 s in a luminometer (CLARIOstar, BMG LABTECH, Germany). The absorbance values were converted into ATP concentration (ng/ μ L) based on ATP standard concentration curve.

2.9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue (CBB) staining

To detect whether the components of bacterial membrane were affected by *B. subtilis* CFS treatment, proteins from *S. aureus* suspension (1×10^8 CFU/ml, 1 mL) pre-treated with PBS (control) or *B. subtilis* CFS were harvested for analysis with SDS-PAGE. The gel was then stained with CBB G-250 to show patterns of protein bands. Whole-cell protein (40 μ g/lane) and membrane protein (70 μ g/lane) were separated with 10% SDS-PAGE. Following electrophoresis, the gel was fixed in a solution of 50% methanol / 10% glacial acetic acid for 6 hours before being stained in the above solution with 0.1% CBB R-250 for 20 m with gentle agitation. Finally, the light blue background of the gel was eluted with destaining solution (40% methanol and 10% glacial acetic acid) before the gel was scanned for further analysis.

2.10 Implant-associated *S. aureus* osteomyelitis mice model

All procedures involving animals were approved by the Animal Care and Use Committee at Nanfang Hospital, Southern Medical University. Male C57BL/6J mice (8–10 weeks old) were obtained from the Animal Center at Southern Medical University. Mice were housed in a facility under specific pathogen-free conditions at 24–27 °C with a 12-h light/dark cycle and had *ad libitum* access to food and water.

The mice model of implant-associated osteomyelitis was made as described previously with modifications [26]. In brief, prior to surgery, they were anesthetized by 125 mg/Kg tribromoethanol (Cat. T831042, Shanghai, China) via intraperitoneal injection. After being shaved and sterilized, an incision was made at the lateral side of the right hind leg and the tibiae was exposed by blunt dissection, and a uni-cortical hole was created at the proximal part of the tibia with a 29-gauge syringe needle. Next, an 8 mm stainless steel pin (0.3 mm in diameter) was inserted into the bone medullary cavity. The hole was sealed with bone wax and the wound was sutured after disinfection. By day 7 post-surgery, *S. aureus* (5×10^7 CFU/mL, 100 μ L) was inoculated by intravenous injection via the tail vein. Mice were monitored twice daily for morbidity and mortality.

To determine the anti-bacterial effect of *B. subtilis* culture CFS *in vivo*, mice with implant-associated *S. aureus* osteomyelitis were injected intraperitoneally with 200 μ L of *B. subtilis* culture CFS or the same volume of PBS (control) every day from the day challenged by *S. aureus*. By days 3 and 14 after *S. aureus* inoculation, the right tibiae were collected aseptically and the implanted stainless steel pin was pulled out for further analysis.

To evaluate the responses of *S. aureus* pre-treated with *B. subtilis* CFS to penicillin in vivo, mice were infected by *S. aureus* (5×10^7 CFU/mL, 100 μ L) pre-treated in 1 ml PBS (control) or *B. subtilis* CFS at day 7 after implantation surgery. The next day after *S. aureus* challenge, mice were intraperitoneally injected with penicillin (80 mg/Kg/d). All the mice were sacrificed at days 3 and 14 post-infection by cervical dislocation and the implanted pins were removed from the bone for bacterial enumeration.

To assess bacterial burden in bone, the right bone infected by *S. aureus* was dissected aseptically free from soft tissue, and homogenized in 1 ml of PBS. A 10-fold dilution of the bone homogenate was plated in TSB agar plate to enumerate the number of CFU. Results of bacterial burden were expressed on a log₁₀ scale. To detect bacterial burden on the implant surface, pins were removed carefully from the tibia after the mice were euthanized. The pins were then sonicated in 1 ml of PBS for 5 min to obtain the biofilm bacteria. Each sample was incubated on TSB agar plates at 37 °C. After 24 h incubation, the number of bacterial colonies was counted.

2.11 Histological analysis and immunofluorescence

To evaluate the pathological changes in bone, paraffin-embedded samples were sectioned in 5- μ m thickness, deparaffinized with xylene and hydrated by ethanol gradient, followed by hematoxylin and eosin (H&E) staining. Quantitative evaluation of the histopathological changes was performed using Smeltzer's scoring methods [27]. The parameters included intraosseous acute inflammation (0–4), intraosseous chronic inflammation (0–4), periosteal inflammation (0–4) and bone necrosis (0–4). A score assigned for each sample was the sum of the scores made from the above 4 parameters by two blinded observers independently.

To detect biofilm *S. aureus* on the implant surface, the pins implanted were removed from the tibia gently by day 14 post infection, rinsed 3 times with PBS and fixed in buffered 4% paraformaldehyde solution for 24 h. The implants were blocked with 3% BSA for 1 h and incubated with the rabbit polyclonal anti-*S. aureus* antibody (Cat. ab20920, Abcam) at 4 °C overnight. On the next day, sections were incubated with 594-conjugated secondary antibody (Cat. 712-586-153, Jackson ImmunoResearch, West Grove, PA, USA). Slides were mounted with antifade mounting medium with DAPI (Cat. S2110, Solarbio, Solarbio Life Sciences, China), and images were acquired with a fluorescence microscope (BX63, OLYMPUS, Japan).

2.12 Scanning Electron Microscopy (SEM)

Steel pins were removed from the tibias at day 14 after *S. aureus* infection before fixed in 2.5% Glutaric dialdehyde at 4 °C for 16 h. After being washed and serially dehydrated in a graded series of ethanol solutions, pins were dried in a critical point dryer (HCP-2; Hitachi, Tokyo, Japan) followed by gold plasma coating (E-1010; Hitachi, Tokyo, Japan). Specimens were imaged using a scanning electron microscope (S-3000N; Hitachi, Tokyo, Japan).

2.13 Statistical analysis

All experiments were performed for at least three times. Since the sample sizes were relatively small and the sample distributions not normally distributed, the nonparametric Mann-Whitney *U* test was applied to compare the differences between the two groups. For comparison of the survival time between the two groups, Gehan-Breslow-Wilcoxon test was used. For assessment of infection rate, Chi-square test was used. $P < 0.05$ was considered statistically significant. All statistical data were analyzed using SPSS 19.0 software.

Results

3.1 *B. subtilis* CFS suppresses the growth of planktonic and biofilm *S. aureus*

The investigation of the effect of *B. subtilis* CFS on the growth of *S. aureus* via measuring the optical density at 600 nm (OD₆₀₀) of planktonic cells at indicated time points showed that *S. aureus* strains grew in tryptic soy broth/*B. subtilis* CFS (TSB/*B. subtilis* CFS) or TSB/ phosphate-buffered saline (TSB/PBS) (control) (Fig. 1A). As seen in Fig. 1B, *B. subtilis* CFS dramatically suppressed the OD₆₀₀ value of *S. aureus* after 4 h of treatment, and the inhibitory effect was even stronger than that of penicillin. Additionally, neither penicillin nor gentamicin treatment of static *S. aureus* culture had an inhibitory effect on formation of biofilm *S. aureus*, whereas *B. subtilis* CFS resulted in a dramatic inhibitory effect on biofilm *S. aureus* (Fig. 1C). To evaluate the effect of *B. subtilis* CFS on biofilm *S. aureus*, the *S. aureus* biofilms were formed on plastic wells after static incubation for 24 h, followed by treatment with *B. subtilis* CFS, penicillin or gentamicin for 8 h (Fig. 1D). Membrane-permeable SYTO9 and membrane-impermeable propidium iodide (PI) staining showed that the biofilm *S. aureus* in the presence of *B. subtilis* CFS exhibited a considerable reduction compared to the control group and groups treated by penicillin or gentamicin (Fig. 1E).

3.2 *B. subtilis* CFS increases antibiotic susceptibility of *S. aureus* in vitro

To evaluate the effect of *B. subtilis* CFS on the response of *S. aureus* to antibiotics, the minimum inhibitory concentration (MIC) of *S. aureus* pretreated with PBS or *B. subtilis* CFS for 1 h was detected using Etest. As shown in Fig. 2A-C, *S. aureus* pretreated with *B. subtilis* CFS had a low pharmacodynamic MIC for both penicillin and gentamicin. Further study showed that *B. subtilis* CFS pretreatment did not suppress the growth of *S. aureus* in TSB, but increased the susceptibility of *S. aureus* to penicillin. *S. aureus* pretreated with PBS started to grow rapidly in TSB with penicillin (0.5 MIC) after 8 h of incubation, whereas the growth of *S. aureus* pre-treated with *B. subtilis* CFS was dramatically suppressed by penicillin after 14 h of incubation (Fig. 2D).

To further examine the effects of *B. subtilis* CFS on antibiotic susceptibility of *S. aureus*, we pretreated *S. aureus* with PBS or *B. subtilis* CFS for 1 h and then performed time-kill assay on planktonic *S. aureus*

exposed to penicillin or gentamicin. As shown in Fig. 2A and 2B, this *S. aureus* strain was not sensitive to penicillin, we therefore made time-kill curves for penicillin at 4 × the MIC of antimicrobial concentration. Significantly decreased cell survival rate was observed in *S. aureus* pretreated with *B. subtilis* CFS compared to control ones after 8 h. Additionally, 99% of *S. aureus* pretreated with *B. subtilis* CFS was killed before 24 h (Fig. 2E). Since this *S. aureus* strain was susceptible to gentamicin, the time-kill curves for gentamicin were made at 2 × the MIC of antimicrobial concentration. Results showed that *S. aureus* pretreated with *B. subtilis* CFS increased its sensibility to gentamicin compared to control ones, 99% of *S. aureus* pretreated with *B. subtilis* CFS was killed before 4 h (Fig. 2F). Based on the above time-kill assay data, the minimum duration for killing 90% (MDK₉₀) values was calculated for *S. aureus* exposed to penicillin or gentamicin. There was a dramatic decrease in MDK₉₀ values of *S. aureus* pretreated with *B. subtilis* CFS than in those of control ones for both penicillin and gentamicin (Fig. 2G). Together, the above data clearly indicated that pretreatment with *B. subtilis* CFS led to a greater sensitivity of *S. aureus* to penicillin and gentamicin.

3.3 *B. subtilis* CFS increases membrane permeability of *S. aureus*

Next, we analyzed the effects of *B. subtilis* CFS on expression of *S. aureus* genes encoding adhesive molecules (Cna and ClfA) and virulence factor Hla, and genes involved in quorum sensing (argA, argB and RNAll) and biofilm formation (lca and sarA). Results showed that *B. subtilis* CFS treatment significantly down-regulated the mRNA expression of all the above genes (Fig. 3A).

Since the permeabilizing property of bacterial cell membrane is pivotal to penetration of antibiotics, we analyzed the membrane integrity of *S. aureus* using SYTO9-PI assay. Results showed that *B. subtilis* CFS disrupted the membrane of *S. aureus* after pre-treatment for 1 h, as evidenced by the presence of PI molecules in *S. aureus* (Fig. 3B).

ATP leakage assays to confirm the effect of *B. subtilis* CFS on the membrane permeabilization showed that *B. subtilis* CFS did not change the whole amount of ATP but significantly increased the levels of extracellular ATP (Fig. 3C), indicating that *S. aureus* membrane was profoundly compromised by *B. subtilis* CFS. Indeed, transmission electron micrographs (TEM) confirmed that *B. subtilis* CFS disrupted the typical semi-rigid structure of *S. aureus*, causing the disruption of cell wall, displacement of cell membrane and extrusion of intracellular content (Fig. 3D).

To evaluate the effect of *B. subtilis* CFS on the membrane proteins of *S. aureus*, whole-cell and membrane proteins of *S. aureus* were detected using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and coomassie brilliant blue (CBB) staining. As seen in Fig. 3E, compared with control, *B. subtilis* CFS treatment considerably changed the pattern of whole-cell protein bands in *S. aureus*. The levels of some proteins decreased while some new proteins appeared. Interestingly, these membrane protein bands with molecular weights of 55, 70 and 100 kDa were much weaker than those of the controls, suggesting that *B. subtilis* CFS has a great effect on the level of proteins in membrane.

3.4 *B. subtilis* CFS reduces a hematogenous implant-associated infection in mice

To test whether *B. subtilis* CFS might protect against *S. aureus* infection *in vivo*, we made a mouse osteomyelitis model of hematogenous implant-associated infection. The groups of mice were infected with 5×10^6 CFU of *S. aureus* at day 7 after surgical implantation. Mice were received PBS (control group) or *B. subtilis* CFS injection once a day from the day challenged by *S. aureus* (Fig. 4A). Treatment of *B. subtilis* CFS improved the survival of mice challenged by *S. aureus* compared with control ones (Fig. 4B). The infection rate in surviving control mice increased between days 3 and 14 post-infection. In contrast, surviving mice had a significantly lower infection rate in *B. subtilis* CFS-treated group compared with those in control group, and the infection rate remained unchanged between days 3 and 14 post-infection (Fig. 4C). Accordingly, enumeration of bacterial burdens revealed that control mice harbored higher bacterial burdens on days 3 and 14 post-infection, while *B. subtilis* CFS treatment did dramatically reduce bacterial burdens in the tibias and implants (Fig. 4D, E).

To detect the effect of *B. subtilis* CFS on growth of biofilm *S. aureus* and changes in bone marrow surrounding an implant, the implants and tibias were harvested on day 14. Immunofluorescence staining showed a considerable amount of *S. aureus*-positive staining on the implant surface in PBS-treated mice, while no obvious signals were observed on the implants in *B. subtilis* CFS-treated mice (Fig. 5A). SEM analysis confirmed biofilm formation rescued by *B. subtilis* CFS treatment (Fig. 5B). Additionally, histologic assessment using hematoxylin and eosin (H&E) staining revealed deformation of bone structure and marked abscess formation within the marrow cavity around the implant in PBS-treated control mice, with no obvious bone destruction in *B. subtilis* CFS-treated mice (Fig. 5C). Histological scores confirmed significantly improved bone structure in the bone of *B. subtilis* CFS-treated mice (Fig. 5D).

3.5 *S. aureus* pretreated by *B. subtilis* CFS is susceptible to penicillin *in vivo*

To address the effect of *B. subtilis* CFS pretreatment on the susceptibility of *S. aureus* to penicillin *in vivo*, we examined the outcomes of penicillin treatment of mice infected by PBS-pretreated or *B. subtilis* CFS-pretreated *S. aureus*. Penicillin treatment did not extend the survival of mice infected by PBS-treated *S. aureus* but significantly prolonged the survival of mice infected by *B. subtilis* CFS-pretreated *S. aureus* (Fig. 6A). In surviving mice, penicillin significantly suppressed the infection rate in mice infected by *B. subtilis* CFS-pretreated *S. aureus* (Fig. 6B). Furthermore, enumeration of *S. aureus* cells in the tibias and implants by days 3 and 14 post-infection showed that the surviving mice infected by *B. subtilis* CFS-pretreated *S. aureus* had significantly decreased bacterial burdens in the infected tibias and implants (Fig. 6C, D). Together, these data supported an increased susceptibility of *S. aureus* pretreated by *B. subtilis* CFS to penicillin.

Discussion

S. aureus is one of the important pathogens causing various infections like osteomyelitis. It is hard to cure, in part because of the ability of *S. aureus* to enter into an antibiotic-tolerance state and the formation of biofilm *S. aureus*. The present study provided evidence for bactericidal effect of *B. subtilis* CFS on *S. aureus* *in vitro* and *in vivo*. We also demonstrated that *B. subtilis* CFS treatment increased the susceptibility of *S. aureus* to penicillin and gentamicin, which might have been associated with changes in membrane components and increased membrane permeability in *S. aureus*, respectively. Furthermore, our findings also demonstrated the sensitivity of *B. subtilis* CFS-pretreated *S. aureus* to penicillin in a mouse model of implant-associated osteomyelitis.

Several studies have reported that *B. subtilis* exerts an antimicrobial effect against a broad spectrum of pathogens through direct bactericidal activity or indirect enhancement of immune response, such as interrupting quorum-sensing regulatory system by production of fengycins [21], inhibiting *S. aureus* adhesion and biofilm formation by production of surfactins [28], and enhancing anti-microbial function of macrophage [29]. In agreement with the above reports, our study has confirmed a potent inhibitory capacity of *B. subtilis* CFS against both planktonic and biofilm *S. aureus* *in vitro*, which may dramatically suppress expression of genes associated with *S. aureus* adhesion, biofilm formation, quorum-sensing and virulence. Furthermore, our data demonstrate the bactericidal effect of *B. subtilis* CFS on biofilm *S. aureus* in a mouse model of implant-associated osteomyelitis.

A critical finding in this study is that *B. subtilis* CFS increased the susceptibility of *S. aureus* to penicillin *in vitro* and *in vivo*. Generally, *S. aureus* strains are found to be resistant to almost all β -lactam antibiotics as they produce β -lactamase that breaks down β -lactam ring or a penicillin-binding protein called PBP2a that has a low binding affinity to β -lactam antibiotics [14, 30]. Our data show that *B. subtilis* CFS suppresses the expression of proteins with molecular weights ranging from 55 to 100 kDa. Since PBP2a has a molecular weight of around 70 kDa and β -lactamase a molecular weight of 36 kDa [31, 32], it is possible that *B. subtilis* CFS may suppress the expression of β -lactamase and PBP2a, thereby increasing the sensitivity of *S. aureus* to penicillin. Additionally, due to the increased membrane permeability of *S. aureus* as detected by SYTO 9/PI staining and ATP leakage assay, *B. subtilis* CFS may also sensitize *S. aureus* to gentamicin, an antibiotic that inhibits protein synthesis.

Increasing evidence has pointed to the importance of functional membrane microdomains in the combat against antibiotic resistance in *S. aureus* and perturbation of functional membrane microdomains assembly may disable bacterial antibiotic resistance [13, 33]. The antimicrobial drugs approved generally target only a fraction of proteins that are involved in membrane or cell wall synthesis [34, 35]. In the present study, *B. subtilis* CFS treatment has been shown to suppress the expression of a bunch of membrane proteins, indicating possible destruction of functional membrane domains in *S. aureus*. Our TEM data supports this mechanism that *B. subtilis* CFS treatment may induce the disruption of cell wall in *S. aureus*.

Conclusion

We have discovered that *B. subtilis* CFS has a potent bactericidal effect against *S. aureus* and may increase its antibiotic susceptibility as well. Although the key components of *B. subtilis* CFS that play an antimicrobial role and the precise mechanism by which *B. subtilis* CFS increases *S. aureus* susceptibility to penicillin require further experimentation, our data strongly suggest that *B. subtilis* CFS may be a promising candidate for novel anti-infective targeted strategies.

Abbreviations

S. aureus, *Staphylococcus aureus*; *B. subtilis*, *Bacillus subtilis*; *B. subtilis* CFS, *B. subtilis* cell-free supernatant; TSB, Tryptic soy broth; PBS, Phosphate-buffered saline; OD, optical density; CFU, Colony forming unit; PI, Propidium iodide; MIC, Minimum inhibitory concentration; MDK₉₀, Minimum duration for killing 90%; qRT-PCR, quantitative real-time PCR; TEM, Transmission Electron Microscopy; ATP, Adenosine 5'-triphosphate; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue; H&E, Hematoxylin and eosin; SEM, Scanning electron microscopy; ANOVA, Analysis of variance.

Declarations

Author contributions

Fan Zhang and Bowei Wang contribute equally to this work.

XZ and FZ designed the experiments; FZ, BW, SL, YL and ZL performed the experiments; FZ and BW analyzed the data, FZ drafted the manuscript; XZ and BY supervised the experiments, revised and approved the manuscript.

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Conflicts of Interests

The authors declare no conflicts of interest.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

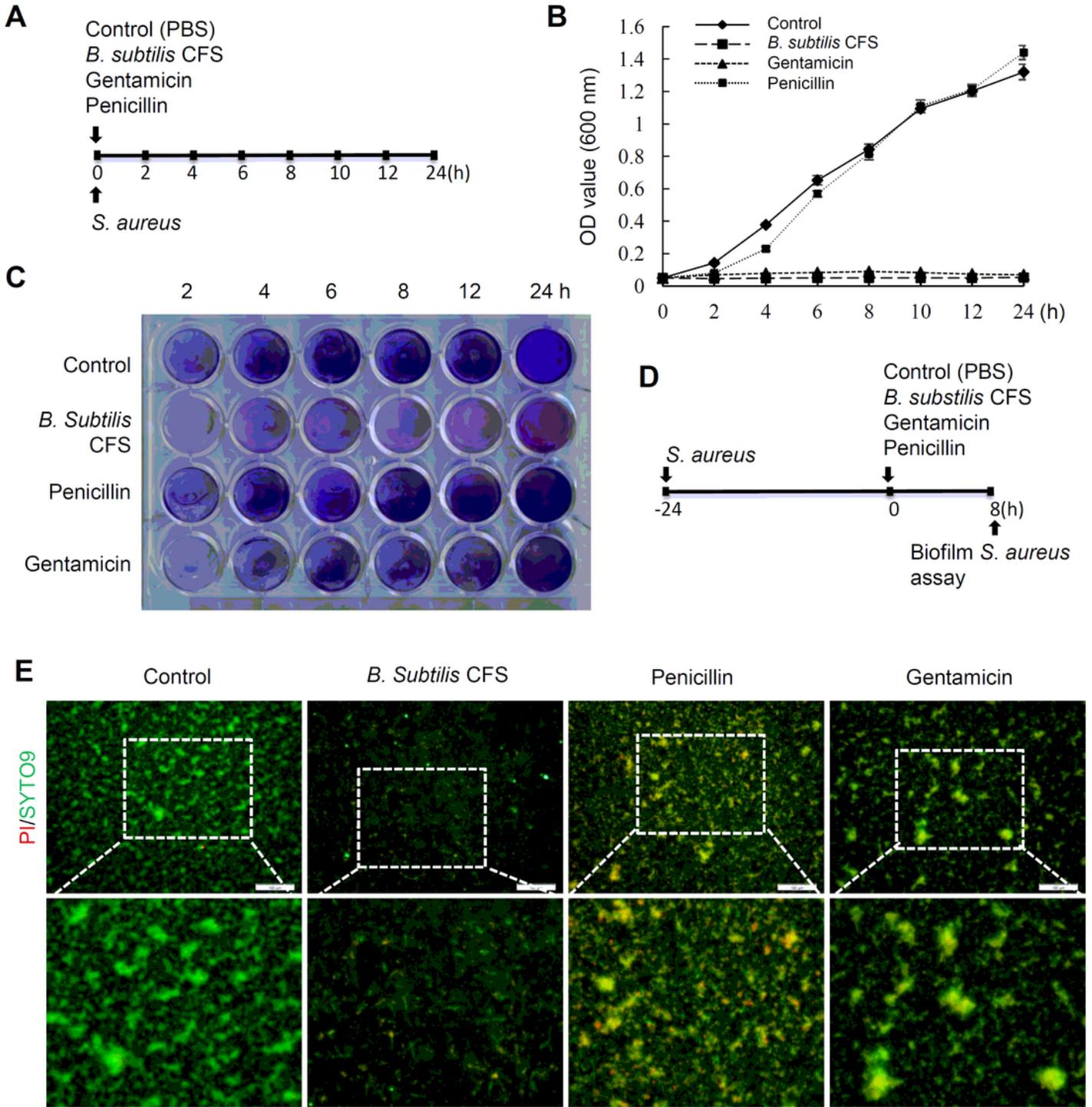


Figure 1

Bacillus subtilis cell-free supernatant (*B. subtilis* CFS) inhibits the growth of planktonic and biofilm *Staphylococcus aureus* (*S. aureus*). (A) Schematic diagram showing the effect of *B. subtilis* CFS treatment on the growth of planktonic *S. aureus*. (B) *S. aureus* (5×10^8 CFU/mL, 100 μ L) was grown in TSB/PBS (control), TSB/*B. subtilis* CFS, TSB/PBS with gentamicin (0.75 μ g/mL), or TSB/PBS with penicillin (32 μ g/mL). Samples were taken out for OD600 evaluation at indicated time points. Data are

shown as mean \pm SE (n = 4 biologically independent samples per time points). (C) Representative images of crystal violet staining for biofilm *S. aureus*. Experiments were repeated independently from 4 different colonies of *S. aureus*. (D) Schematic diagram showing the effect of *B. subtilis* CFS treatment on biofilm *S. aureus*. (E) Representative images of SYTO9-PI staining for biofilm *S. aureus*. *S. aureus* (5×10^7 CFU/mL) was grown at 37°C for 24 h, and then treated with TSB/PBS (control), TSB/*B. subtilis* CFS, TSB/PBS with gentamicin (0.75 μ g/mL), or TSB/PBS with penicillin (32 μ g/mL) for 8 h. Biofilm *S. aureus* was examined with SYTO9-PI, followed by analysis using a fluorescence microscope. Live and dead bacteria were stained green from SYTO9, and dead ones red from PI. Scale bar 100 μ m.

Figure 2

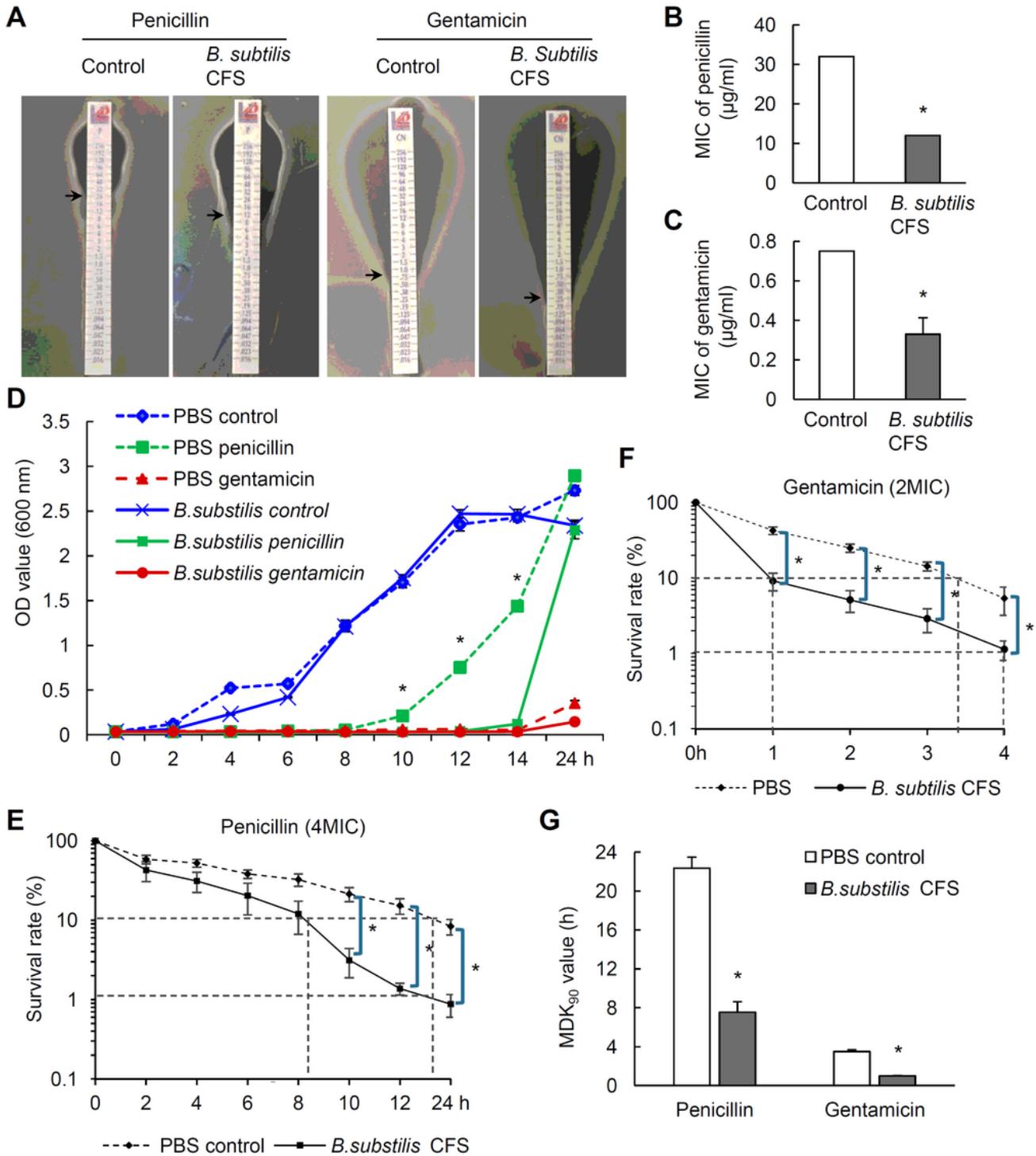


Figure 2

B. subtilis CFS increases *S. aureus* susceptibility to penicillin and gentamicin. (A) Representative Etest images of *S. aureus* from penicillin and gentamicin. The minimum inhibitory concentration (MIC) was read off of the strip where the bottom portion of the ellipse intersects with the strip (see black arrows). The MICs of penicillin to PBS-treated *S. aureus* (control) and *B. subtilis* CFS-treated *S. aureus* were 32 µg/ml and 12 µg/ml, respectively. The MICs of gentamicin to PBS-treated *S. aureus* (control) and *B.*

subtilis CFS-treated *S. aureus* were 0.75µg/ml and 0.25 µg/ml, respectively. (B and C) Quantitative analysis show dramatically decreased MICs of penicillin and gentamicin against *S. aureus* pretreated by *B. subtilis* CFS. MIC values were measured using aliquots of *S. aureus* cultures from three different colonies. *P < 0.05, Mann-Whitney U test. (D) The growth of *S. aureus* pretreated with PBS or *B. subtilis* CFS were monitored with or without the presence of penicillin or gentamicin. Fresh overnight culture of *S. aureus* (5×10^7 CFU/mL) was pretreated with PBS or *B. subtilis* CFS for 1 h, and then challenged with PBS, 0.5 MIC penicillin or gentamicin. Samples were collected and OD600 was recorded at indicated time points. N=4/group at each time point. *P < 0.05, Mann-Whitney U test. (E and F) Time-dependent killing of control *S. aureus* and *B. subtilis* CFS-pretreated *S. aureus* by penicillin at 4×MIC (E) and gentamicin at 2×MIC (F). Experiments were independently repeated for 4 times. *P < 0.05, Mann-Whitney U test. (G) Minimum duration for killing 90% (MDK) measurements of control *S. aureus* and *B. subtilis* CFS-pretreated *S. aureus* exposed to penicillin at 4×MIC or gentamicin at 2×MIC. Values were determined from the quadruplicate data shown in (shown in E and F). *P < 0.05, Mann-Whitney U test.

Figure 3

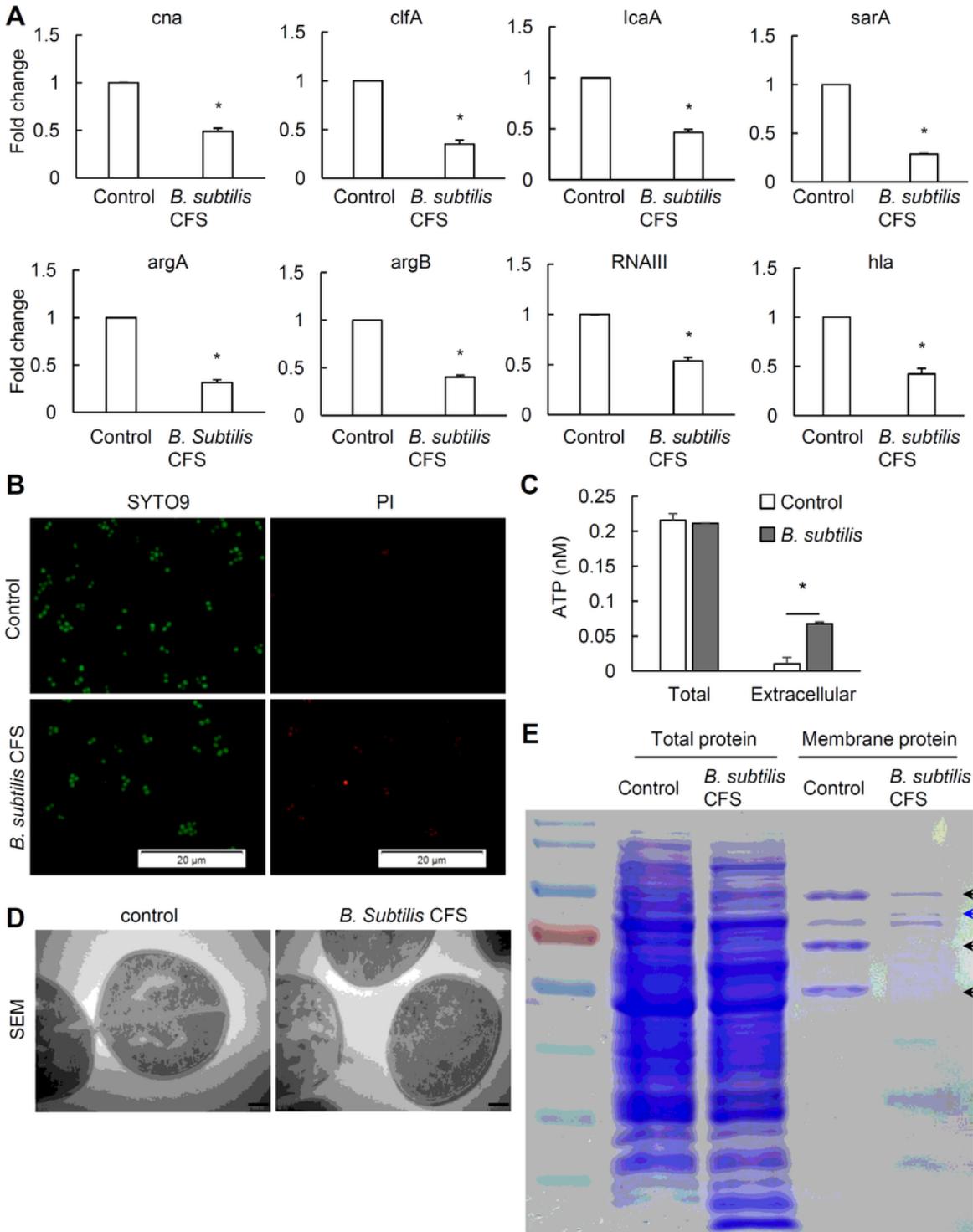


Figure 3

B. subtilis CFS alters the pattern of genes expression and increases membrane permeability of *S. aureus*. (A) qRT-PCR analysis of the genes involved in adhesive molecules (Cna and ClfA), virulence factor Hla, quorum sensing (*argA*, *argB* and *RNAIII*) and biofilm formation (*lca* and *sarA*) in *S. aureus*. *S. aureus* (1×10^8 CFU/mL) was treated with PBS (control) or *B. subtilis* CFS for 3 h. $N = 4$ /group, $*P < 0.05$, Mann-Whitney U test. (B) Representative images of SYTO9-PI staining to detect the effect of *B. subtilis* CFS on

the membrane permeability of *S. aureus*. *S. aureus* (1×10^8 CFU/mL) was treated with PBS (control) or *B. subtilis* CFS for 1 h, followed by staining with 10 μ M of SYTO9 (membrane-permeable) and 3 μ M of PI (membrane-impermeable). Live and dead cells were stained with green, and dead ones stained red. Scale bar 20 μ m. (C) The leakage of cellular ATP from *S. aureus* after treatment with *B. subtilis* CFS. Data are represented as means \pm SD of 4 independent colonies. * $P < 0.05$ vs control. Mann-Whitney U test. (D) Representative TEM images of PBS-treated (control) and *B. subtilis* CFS-treated *S. aureus*. Control *S. aureus* cells showed even cell walls but *B. subtilis* CFS-treated *S. aureus* compromised cell walls. Scale bar 100 nm. (E) Representative images of Coomassie brilliant blue (CBB) staining for whole-cell and membrane proteins of PBS-treated (control) and *B. subtilis* CFS-treated *S. aureus*. Black arrows indicate decreased levels of proteins at 55, 70 and 100kDa, and the blue arrow increased level of proteins. Experiments were repeated independently from 4 different colonies of *S. aureus*.

Figure 4

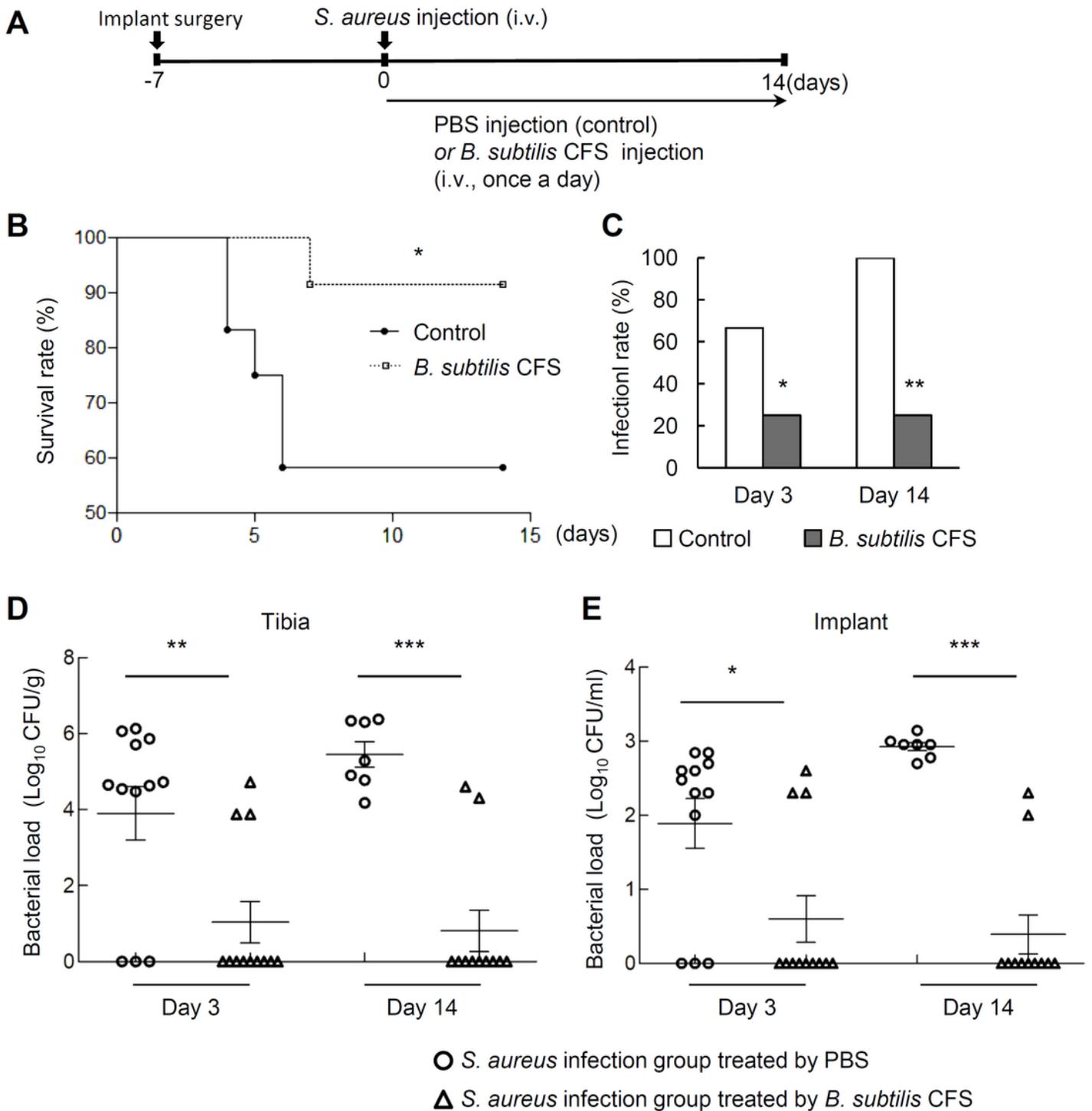


Figure 4

B. subtilis CFS suppresses *S. aureus* burden in a mouse model of implant-associated osteomyelitis. (A) Schematic diagram showing establishment of implant-associated *S. aureus* osteomyelitis in mice and treatments. After challenged with *S. aureus*, mice were treated daily with *B. subtilis* CFS or the PBS control. Colony forming unit (CFU) of *S. aureus* was enumerated from the implanted-tibia on days 3 and 14. (B) Survival of osteomyelitis mice treated with PBS (control) and *B. subtilis* CFS. Data represent

aureus on the implant surface. Experiments were repeated independently from 4 samples per group. Blue scale bar 200 μm and black scale bar 10 μm . (C) Representative images for hematoxylin and eosin (H&E) stained tibial sections from the osteomyelitis mice treated with PBS or *B. subtilis* CFS on day 14 post-infection. Scale bar 500 μm . (D) Histological assessment of H&E stained sections. $N = 6/\text{group}$, $*P < 0.05$ vs control. Mann-Whitney U test.

Figure 6

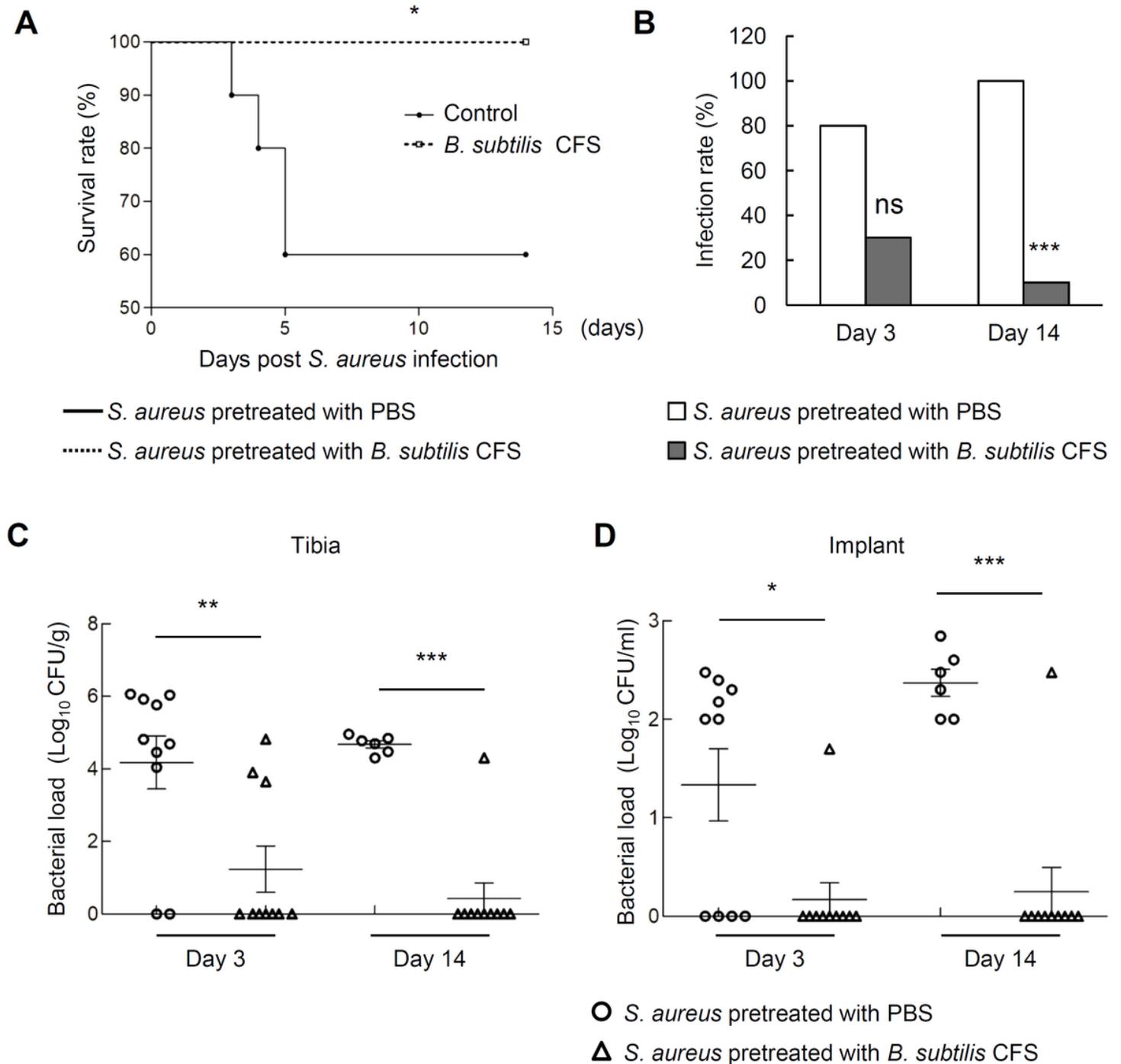


Figure 6

Bacillus subtilis cell-free supernatant (B. subtilis CFS) increases the susceptibility of S. aureus to penicillin. (A) Survival percentage of implant-associated osteomyelitis mice infected by S. aureus pretreated with PBS (control) and B. subtilis CFS. All mice were treated with penicillin (80 mg/Kg/d) from the day challenged by S. aureus. N = 10/groups, *P < 0.05, Gehan-Breslow-Wilcoxon test. (B) Infection rate in surviving mice infected by S. aureus pretreated with PBS (control) and B. subtilis CFS on days 3 and 14 post-infection. N=10/group, *P < 0.05, ns P > 0.05, Chi-square test. (C+D) Quantification of S. aureus loading recovered from the implanted-tibia (C) and the needle (D) on days 3 and 14 post-infection. N = 10/group, **P < 0.01, ***P < 0.001, Mann-Whitney U test.

Supplementary Files

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- [Table1.tif](#)