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Octanoic acid promotes clearance of antibiotic-tolerant cells and eradicates biofilms of *Staphylococcus aureus* isolated from recurrent bovine mastitis

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ABSTRACT

Antibiotic therapy is the primary treatment for bovine mastitis, but the drawbacks of this strategy include poor cure rate and economic losses from the need to discard milk with antibiotic residues. Unfortunately, few other treatment options are currently available for mastitis. Failure of antibiotic treatments is often attributed to formation of bacterial biofilms and abscesses in the mammary gland tissue, which lead to chronic infections that are difficult to eradicate and drive recurrent disease. A major mastitis-causing pathogen (MCP) associated with biofilms in bovine mastitis is *Staphylococcus aureus*. In this study, we demonstrate that octanoic acid has broad-spectrum microbicidal activity against MCPs and effectively inhibits *S. aureus* biofilm formation in milk (>50% inhibition at 3.13 mM). Octanoic acid effectively clears biofilms (95% eradication at 1*X* minimum bactericidal concentration, MBC) and infrequently induces *S. aureus* small colony variants (SCVs) that may cause recurrent mastitis. Additionally, octanoic acid rapidly kills persistent biofilm cells and cells with antibiotic tolerance (within 4 h). In contrast, antibiotics treated at >100*X* MBC cannot eradicate biofilms but do induce SCVs and antibiotic-tolerant cells. These effects may accelerate the transition from biofilm to chronic infection. Thus, octanoic acid exhibits bactericidal action against *S. aureus* biofilms, and it is less likely than antibiotic therapy to induce persistent cells and pathogen tolerance. Moreover, octanoic acid acts additively with antibiotics against *S. aureus*, and it attenuates tetracycline-induced virulence factor gene expression in *S. aureus* cells. According to these data, octanoic acid may prevent the pathological progression of bovine mastitis and offer a new strategy for treating the condition.

1. Introduction

Bovine mastitis is a costly and highly prevalent disease in the dairy industry, which reduces the yield and quality of milk. Raw milk from cows with mastitis typically has a high total bacterial count and somatic cell count, resulting in lower quality and purchase price [1,2]. The standard response to clinical mastitis in dairy cattle involves early detection, isolation of infected cows, administration of antibiotics, use of anti-inflammatory drugs, provision of supportive care, and culling if necessary. To mitigate the negative impacts of this disease, dairy farms take great care to control infection and inflammatory responses in the cows. Animals that develop mastitis are primarily treated with intra-mammary antibiotics to eliminate the bacteria that cause udder inflammation [2,3]. However, these antibiotic treatments suffer from low cure rates [1] and are associated with a high risk of chronic infections [4,5]. Moreover, raw milk with antibiotic residues must be discarded, leading to economic losses.

Bovine mastitis-causing pathogens (MCPs) are generally classified as contagious or environmental according to their transmission modes [6–9]. Environmental pathogens are responsible for a relatively small

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portion of infections (less than 10% of total mastitis cases) and mainly affect cows with low immunity or that reside on farms with inadequate hygiene. In contrast, contagious pathogens like *Staphylococcus aureus* and *Streptococcus agalactiae* are typically transmitted through direct contact with cows that have infected udders or via milking machines [2,6,7]. *S. aureus* colonization and penetration of mammary cells often results in biofilms or abscesses that protect the bacteria from antibiotics and immunocytes. These characteristics make it challenging to eradicate the bacteria, leading to low cure rates, bacterial persistence, and development of antimicrobial resistance [1,2,6,7]. Persistent or recurrent infection may further cause damage to mammary tissue that can decrease or even completely stop milk production [1].

Early detection and treatment of infected cows are critical to preventing disease spread. Since identifying pathogenic bacteria can take several days and cows are at risk of transmission during this time, broad-spectrum antibiotics are often applied immediately upon signs of infection [6]. However, widespread application of this strategy results in the overuse or misuse of antibiotics [1,3], which contributes to the global public health concern of antimicrobial-resistant pathogens [3,4]. Excessive use of antibiotics may induce the development of persister cells, which can be identified by their altered transcriptomic profiles and contribute to relapsing infections and therapeutic failure [4,5]. Therefore, alternative therapies for bovine mastitis are urgently needed.

Octanoic acid is a saturated fatty acid present in natural food sources, such as coconut, palm kernel and milk [10]. It exhibits antibacterial activity against various species, including *S. aureus*, *Escherichia coli* and *Candida albicans* [11–17]. Previous studies have shown that octanoic acid causes bacterial cell death by disrupting the cell membrane [14,17], and hinders microorganisms' growth and proliferation by impairing metabolism [17]. Furthermore, several studies have demonstrated that octanoic acid exhibits anti-inflammatory properties [18,19]. Based on those characteristics, octanoic acid may be a promising candidate for bovine mastitis therapy. However, evidence for its utility in treating bovine mastitis and preventing development of chronic infection is lacking. This study addressed this issue using a biofilm model of *S. aureus* isolated from recurrent clinical mastitis. We evaluated the efficacies of octanoic acid and standard antibiotics in eradicating bacterial biofilms. Our findings are consistent with the low cure rate for antibiotics and suggest that octanoic acid may have a better potential for preventing chronic infection in bovine mastitis.

2. Materials and methods

2.1. Bacterial strains and culture

A total of 22 clinical isolates were used in this study, as listed in Table S1. *Staphylococcus aureus* ATCC 12600 was purchased from the American Type Culture Collection (ATCC). All clinical isolates were obtained from the raw milk of dairy cows with mastitis. Organisms were identified by 16S rRNA gene sequencing. The definition of each clinical isolate number is given in Table S1. Udders 8 to 13 had recurrent clinical mastitis that was treated with Ceroxim No. 2 ointment (Fig. S1A). A veterinarian confirmed mastitis through mammary histopathology and tissue biopsy, which also revealed abscess formation in the tissues (Fig. S2). Six *Streptococcus* spp. isolates (i.e., 1-1, 2-1, 2–6, 3-2, 6-2 and 9-6; Table S1) were cultured in Tryptone Soy Agar (TSB, Difco BD) with 5% horse serum (Gibco), and the remaining 17 isolates were cultured in TSB without horse serum. All 23 isolates were cultured for 16 h at 37 °C shaking incubator with 200 rpm.

2.2. Microbroth dilution assays

Bovine mastitis is generally treated with antibiotics such as beta-lactams and tetracyclines. Therefore, we evaluated the susceptibility of MCPs to octanoic acid and five commonly used bovine mastitis antibiotics, including ampicillin, cloxacillin, cefuroxime, cefotaxime and tetracycline (Sigma-Aldrich). The antimicrobial activity of octanoic acid and antibiotics was evaluated according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Mueller Hinton II broth (MHB) was used for microbroth dilution assays of all pathogens except *Streptococcus* spp., which were tested in MHB with 5% horse serum. Antimicrobial agents were serially diluted in 96-well round-bottom polystyrene plates (Corning) with culture medium to generate various concentrations of antibiotics (0–100 μg/mL) and octanoic acid (0–200 mM). A final inoculum of 5×10^5 colony-forming units (CFU)/mL was produced by adding an appropriate amount of overnight bacteria culture. The MIC was defined as the minimal inhibitory concentration of an antimicrobial agent with no visible growth after 24 h incubation. Bacteria were subcultured from the MIC test broth to TSA plates, and the minimum bactericidal concentration was defined as the MBC. The MBC in milk was measured by the same protocol as described above, with the only difference being the replacement of medium with milk (Kuang Chuan, Taiwan).

2.3. Synergy checkerboard assay

The antimicrobial activities of octanoic acid combined with different antibiotics (ampicillin, cloxacillin, cefuroxime, cefotaxime, and tetracycline) were tested on *S. aureus* ATCC 12600 and clinical isolate 10-9. Serial two-fold dilutions of octanoic acid and antibiotics were made to generate various concentrations. The final inoculum was generated by adding fresh overnight bacterial culture to 5×10^5 CFU/mL. After a 24-h incubation, the MIC of each combination was determined by measuring optical density at 600 nm (OD600). FIC indices (FICI) were defined according to a published protocol [20].

2.4. Biofilm formation assays

The biofilm-forming capacity of each bacterial isolate was determined according to a previously described method with necessary modifications [21,22]. All pathogens were grown in sBHI (referred to as biofilm medium in the figures), which consists of BHI containing 0.3% starch and 1% glucose. A 200 μL overnight culture of bacteria at 1×10^6 CFU/mL was added in sBHI or milk to 96-well flat-bottom plates. Planktonic cells were removed after 24 h incubation, and each well was rinsed twice with saline. Following the washing step, the plates were oven-dried for 1 h. Biofilms were visualized by staining with a 0.5% crystal violet solution. The plates were washed with saline to remove the unbound dye, and biofilm-forming capacity was determined by optical density measurement at 585 nm (OD585). The inhibitory activity of octanoic acid on biofilm formation was assessed by incubating bacteria with serial dilutions of octanoic acid in sBHI or milk for 24 h, followed by quantification of biofilm.

2.5. Biofilm cell viability assay

An overnight culture of *S. aureus* ATCC 12600 was added to 200 μL sBHI at 1×10^6 CFU/mL in 96-well flat-bottom plates. Planktonic cells were removed after 16 h incubation at 37 °C. Various concentrations of octanoic acid (0–100 mM) or antibiotics (0–400 μg/mL) were added to the mature biofilms. The commercial antibiotics, Maxalac LC and Ceroxim No. 2 ointment (Fig. S1), were purchased from a local pharmacy. Ointments were serially diluted in sBHI and added to mature biofilms (dilutions were made from 1:8 to 1:256 in order to achieve the desired concentration range). Biofilms were incubated with drugs for 24 h at 37 °C. Then, bacterial viability was analyzed using alamarBlue (Invitrogen) according to the manufacturer's instructions.

2.6. Scanning Electron Microscopy (SEM)

S. aureus biofilm cell morphology was observed by SEM after octanoic acid or antibiotic treatments. The biofilm to be tested was cultured

on a plastic film and fixed with 4% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (PBS), pH 7.0, at room temperature for 1 h. Biofilms were rinsed three times for 10 min with 0.1 M PBS and then fixed with 2% osmium tetroxide in PBS at room temperature for 1 h. The samples were then dehydrated through an alcohol concentration gradient (30%–100%) for 10 min at each concentration. Critical point drying was performed with a Leica EM CPD300 critical point dryer. A Hitachi E−1010 ion sputter was used for sample coating. A FEI Quanta 200 environmental SEM at 20 KV was used for observation and imaging.

2.7. Confocal laser-scanning microscopy (CLSM)

S. aureus ATCC 12600 mature biofilm was treated with 50 mM octanoic acid or 0.4 mg/mL antibiotic. After 16 h of incubation at 37 °C, biofilm cells were stained with the LIVE/DEAD BacLight kit (Invitrogen) according to the manufacturer's instructions. Fluorescence-adherent bacteria were directly observed using a CLSM (FV3000, Olympus).

2.8. Evaluation of colony variance during S. aureus biofilm upon antibiotic exposure

Mature biofilms of *S. aureus* isolate 10-9 were treated with 25 mM octanoic acid or 0.4 mg/mL antibiotic (ampicillin, cloxacillin, cefuroxime, cefotaxime, and tetracycline). The treatment drugs and medium (sBHI) were replaced every 24 h until the third day to simulate repeated administration to infected animals. Each day the bacterial viability was determined by the CFU plate-counting method. SEM analysis was performed to monitor morphological changes in the biofilm cells on the first day after drug treatment; SCVs were identified based on the reference description [23]. Biofilm cells were then plated on LB agar plates on the fourth day after drug treatment. SCVs were defined as colonies at least 10-fold smaller than normal colonies [23,24].

2.9. RNA isolation and quantitative real-time PCR

Total RNA was isolated according to the manufacturer's directions using the TRIzol max bacterial RNA extraction kit (Invitrogen). Reverse transcription of total RNA was performed with ReverTra Ace® qPCR RT Master Mix (Toyobo). The transcript levels of the *fnbA, clfA, agrA, rbf, sarA, arlR, hla,* and *sigB* genes were determined using quantitative real-time PCR (qRT-PCR) and an ABI StepOnePlus Real-Time PCR Instrument (Applied Biosystems, Foster, CA, United States). Table S2 lists the primers used for qRT-PCR. The gene expression levels were calculated using the $2^{-\Delta Ct}$ method, with 16S rRNA expression serving as the endogenous control. The expression level fold-change was calculated relative to the 0 h untreated group.

2.10. Time-kill analysis for definitions of antibiotic resistance, tolerance and persistence

Cell persistence and antibiotic tolerance were evaluated according to published protocols [25]. The definition of tolerance was an increase in the minimum duration to kill 99% of bacteria (MDK99), and persistence was an increase in the minimum duration to kill 99.99% of bacteria (MDK99.99) compared with susceptible bacteria. *S. aureus* ATCC 12600 and isolate 10-9 were cultured in TSB and grown at 37 °C for 16 h under shaking at 200 rpm to produce cells at the planktonic stage. Biofilms were formed using cultures in sBHI. Medium was removed, and planktonic cells were left to develop into biofilm stage. Mature biofilms were treated with 0.4 mg/mL tetracycline or cloxacillin for 16 h, then centrifuged at 8000×*g* and washed with saline to eliminate the antibiotics. Collected cells were subjected to antibiotic exposure at approximately 10^5 CFU/mL for time-kill analyses. Bacterial cells were incubated with 2 × MBC octanoic acid and 4 × MBC antibiotics. The CFUs were counted from cultures at 0, 4, 8 and 24 h.

2.11. Statistical analysis

Data were collected from at least three independent experiments, and statistical analyses were performed using GraphPad Prism 8.0 software. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Octanoic acid has broad-spectrum microbicidal activity against MCPs and shows additive effects with antibiotics

In total, 22 clinical isolates were collected from raw milk derived from 12 cows with udders that had mastitis (Table S1, excluding strains that were difficult to culture, including *Aerococcus* spp., *Acidovorax* spp.). *Staphylococcus* spp. was the most commonly isolated type of MCP (40.9%, 9 out of 22 isolates), followed by *Streptococcus* spp. (6/22) and *E. coli* (3/22). Isolation of these bacteria is consistent with mastitis-associated pathogens identified in previous studies [26,27]. Udders 8 to 13 had recurrent clinical mastitis that had been treated with Ceroxim No. 2 ointment. The major pathogens isolated from these udders were *Staphylococcus* spp., which are commonly associated with mastitis recurrence. Most *Staphylococcus* spp. and *Streptococcus* spp. were sensitive to the tested antibiotics (Fig. 1A and B and Fig. S3). However, all *E. coli* (7–13, 8-8 and 9-8) resisted cloxacillin. *Enterococcus faecium* (5-1 and 8-3) was resistant to almost all tested antibiotics, and 9–12 *Bacillus pumilus* was resistant to cefuroxime and cefotaxime. 12-10 *Acinetobacter towneri* resisted ampicillin and cloxacillin (Fig. 1C and Fig. S3).

In contrast, octanoic acid exhibited bactericidal effects against all MCPs at concentrations less than 100 mM, and it remained effective in milk (Fig. 1D). Next, we combined octanoic acid with antibiotics. Importantly, octanoic acid had additive effects with several antibiotics and synergistic effects with cefuroxime (Fig. 2A and B). Taken together, these findings led us to conclude that *Staphylococcus* spp. were the major pathogens in recurring clinical mastitis, but most of the isolates remained susceptible to traditional antibiotics. Recurrence could therefore be caused by factors unrelated to antibiotic resistance. Most importantly, the data demonstrate that octanoic acid has broad-spectrum microbicidal activity against MCPs and acts additively with antibiotics.

3.2. Octanoic acid effectively inhibits biofilm formation by S. aureus

S. aureus can penetrate mammary gland tissue and form biofilms or abscesses, which are critical factors in the recurrence of antibiotic treatment [28]. Preventing biofilm formation is therefore crucial to halting disease progression toward chronic and recurrent infection. The capacities of each isolate to form biofilms are listed in Table S1. Among all isolates, *Staphylococcus* sp. showed the strongest capacities for forming biofilms. In particular, *S. aureus* 7-2, 7-3, 10-9, 11-1, and 12-12 exhibited high capacities to form biofilms in milk and also formed curds in the milk (Fig. 3A). Other strains could not form or weakly formed biofilms (data not shown). We next investigated the effects of octanoic acid on the biofilm formation capacities of these strains. *S. aureus* ATCC 12600, 10-9, 11-1 and 12-12 formed biofilms in sBHI medium, but the biofilm formation by each of the four *S. aureus* strains was significantly inhibited by treatment with 12.5 mM octanoic acid (>50% inhibited; Fig. 3B). Notably, *S. aureus* strains 7-2, 7-3, 10-9, 11-1 and 12-12 formed solid biofilms in milk but were inhibited by more than 50% when treated with 3.13 mM octanoic acid (Fig. 3C). SEM images showed a clear extracellular matrix (ECM) entangled between *S. aureus* cells in the untreated control. However, the apparent *S. aureus* density and ECM were drastically reduced in octanoic acid-treated samples (6.25 and 12.5 mM) compared to the untreated control (Fig. 3D). These findings indicate that octanoic acid effectively inhibits biofilm formation by *S. aureus* (a robust biofilm-producing MCP), and the inhibitory effect is more potent in milk than in sBHI medium.

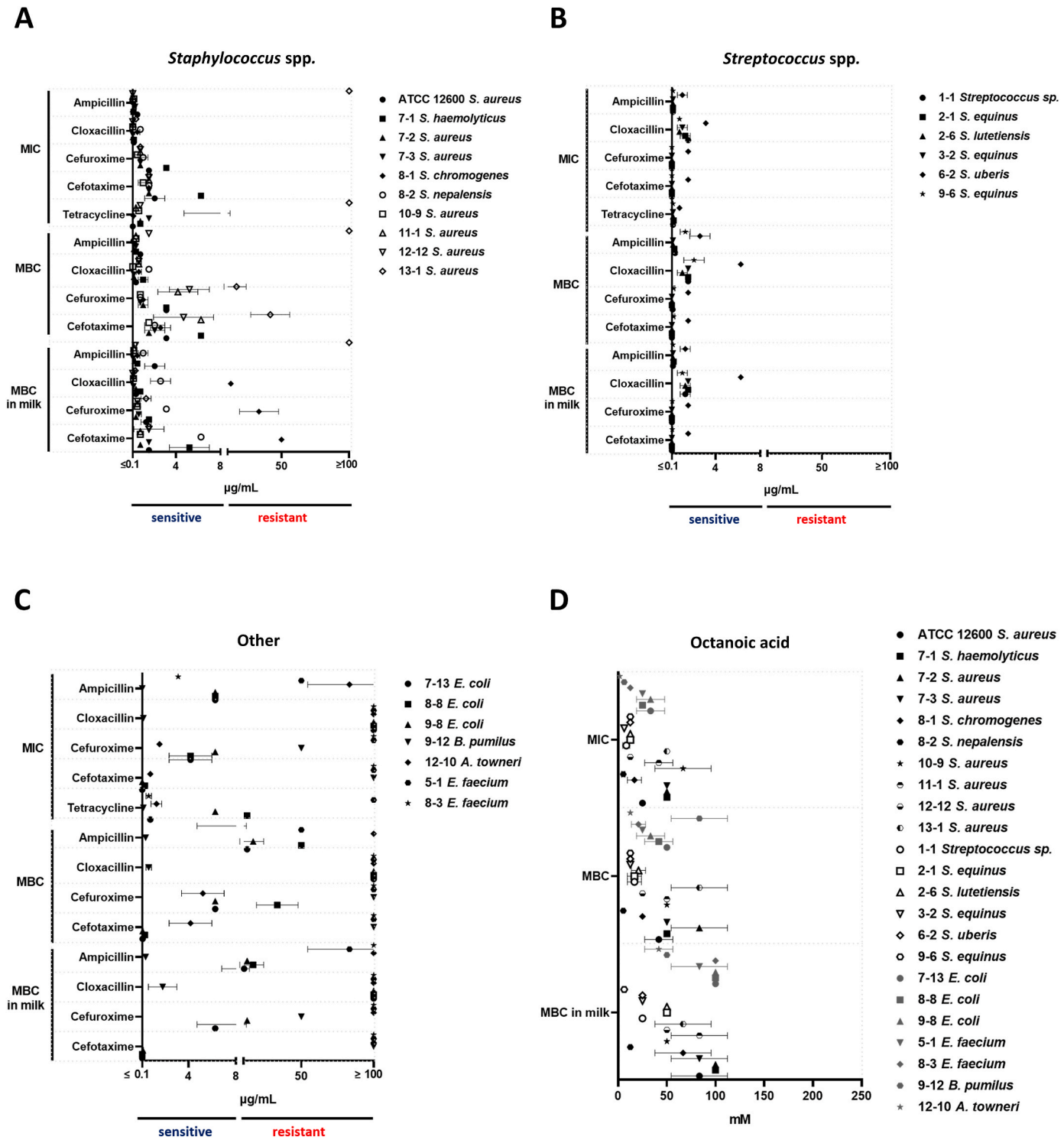
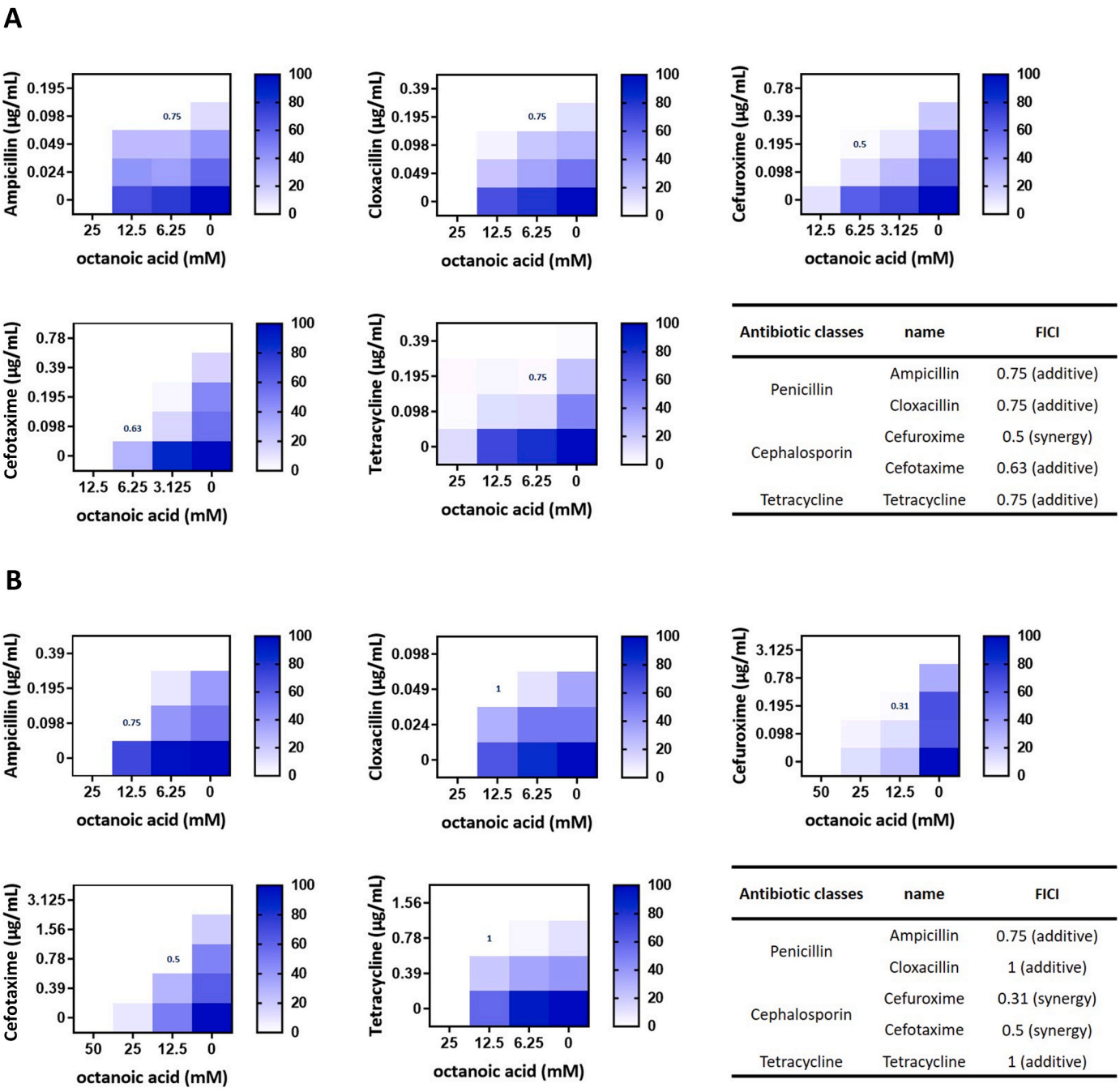


Fig. 1. Antibacterial activity of octanoic acid and antibiotics tested on bovine mastitis pathogens. Antibiotic susceptibility testing in (A) *Staphylococcus* spp., (B) *Streptococcus* spp., (C) *Escherichia coli*, and other pathogens isolated from dairy cows with mastitis. (D) Octanoic acid antimicrobial efficacy against *S. aureus* ATCC 12600 and clinical isolates of MCPs. MICs and MBCs of octanoic acid and antibiotics tested on *S. aureus* ATCC 12600 and clinical isolates of MCPs. The MBCs in milk were also determined in this study. All values represent the mean ± SD of three individual experiments. Determination of susceptibility of strains to antibiotics followed CLSI guidelines, with minor modifications. Susceptibility is defined by a MIC of ≤4 μg/mL and a MIC of ≥16 μg/mL was considered resistant.



Antibiotic classes	name	FICI
Penicillin	Ampicillin	0.75 (additive)
	Cloxacillin	0.75 (additive)
Cephalosporin	Cefuroxime	0.5 (synergy)
	Cefotaxime	0.63 (additive)
Tetracycline	Tetracycline	0.75 (additive)

Antibiotic classes	name	FICI
Penicillin	Ampicillin	0.75 (additive)
	Cloxacillin	1 (additive)
Cephalosporin	Cefuroxime	0.31 (synergy)
	Cefotaxime	0.5 (synergy)
Tetracycline	Tetracycline	1 (additive)

Fig. 2. Antibacterial effects of octanoic acid and antibiotic combinations against bovine mastitis pathogen *S. aureus*. Checkerboard analyses showing percentage inhibition for the combined effects of octanoic acid with ampicillin, cloxacillin, cefuroxime, cefotaxime and tetracycline toward (A) *S. aureus* ATCC 12600 and (B) clinical isolate 10-9. The heat map shows the average of three replicates. The positions of the blue values represent the concentrations calculated by FICI. (F) The FICI results and definitions of octanoic acid and antibiotic combinations. FICI $\leq$ 0.5 indicates synergism, while FICI >0.5–1 indicates an additive effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Octanoic acid is more potent than antibiotics in eradicating S. aureus biofilms

Since mastitis is difficult to detect in the early stages of infection (subclinical mastitis), the optimal therapy window is often missed. Once a biofilm has formed, it becomes challenging to treat. After showing that octanoic acid has broad-spectrum antimicrobial activity against MCPs and effectively inhibits biofilm formation, we next compared the efficacies of octanoic acid and antibiotics in eradicating mature *S. aureus* biofilms. Each tested antibiotic significantly decreased biofilm cell viability at the lowest dose of 12.5 μg/mL, with no additional impact on cell viability observed at higher antibiotic doses. As such, approximately 50% cell viability was maintained across all antibiotic groups, and the biofilms even persisted at doses >100X MBC (Fig. 4A and D). Similar results were obtained with testing commercial intramammary antibiotics Maxalac LC and Ceroxim No. 2 ointment. This result suggests that increasing the dose of antibiotics will not lead to biofilm eradication (Fig. 4B). In contrast, octanoic acid eradicated biofilms in a dose-dependent manner, killing ~95% of biofilm cells at 1X MBC (25 mM) (Fig. 4C and D). SEM images revealed (Fig. 4E) that the *S. aureus* biofilm

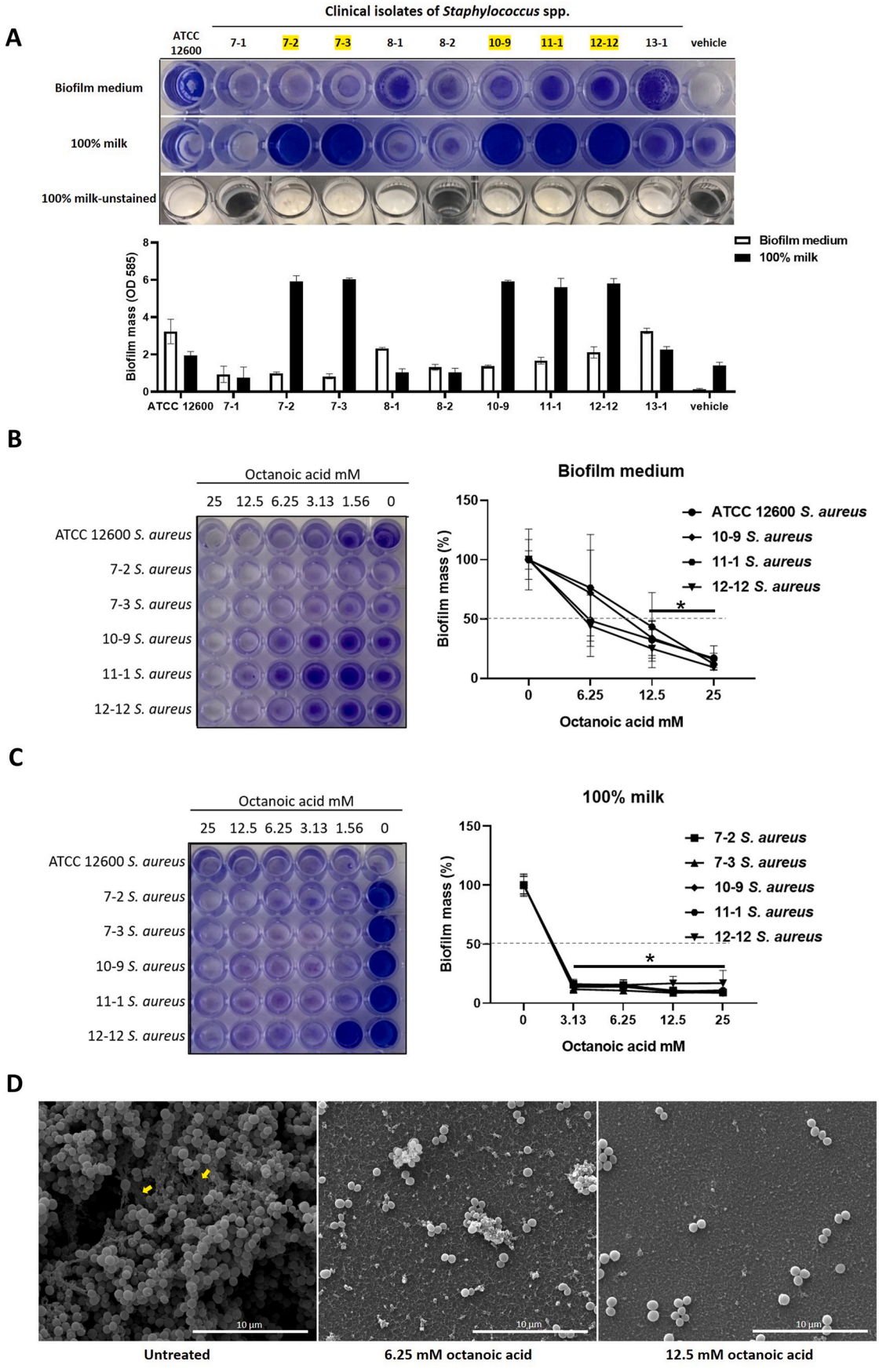


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Fig. 3. Evaluation of biofilm formation inhibition activity of octanoic acid in the recurrence of mastitis major pathogens *S. aureus*. (A) A photograph of *S. aureus* ATCC 12600 and clinical isolates of *Staphylococcus* sp. biofilms stained with crystal violet (upper) and biofilm biomass quantification (lower). Highlighted (yellow) clinical isolates 7-2, 7-3, 10-9, 11-1, and 12-12 are strains with strong biofilm-forming capacity in milk. Biofilms formed by *S. aureus* ATCC 12600 and clinical isolates 7-2, 7-3, 10-9, 11-1, and 12-12 were tested with octanoic acid (0–25 mM) in sBHI (B) and milk (C). After a 24 h incubation at 37 °C, biofilms were stained with crystal violet for quantification of bacterial biomass. Untreated control was taken as 100% biofilm formation. The dotted line represents 50% biofilm inhibition. All values represent the mean ± SD of three individual experiments. $^{*}p < 0.05$ compared with the untreated. (D) SEM images at 12000 × magnification in *S. aureus* ATCC 12600 biofilm formation treatment by octanoic acid (untreated, 6.25 and 12.5 mM). Yellow arrows represent the extracellular matrix of the *S. aureus* biofilm. Scale bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cells in untreated samples were plump, intact with a smooth exterior, and embedded in a viscoelastic ECM. In contrast, the octanoic acid-treated biofilms (25 and 50 mM) exhibited cell collapse, reduced staphylococcal surface attachment, and limited ECM. Fluorescent staining with the LIVE/DEAD BacLight kit showed that dead cells (propidium iodide/red) were much more common in the octanoic acid-treated *S. aureus* biofilms than in antibiotic-treated biofilms (Fig. 4F). These findings suggest that octanoic acid greatly outperforms available antibiotics in terms of biofilm eradication.

3.4. Treatment of biofilms with octanoic acid induces fewer SCVs than antibiotics

Administering multiple doses of antibiotics is common practice for mastitis. However, the repeated treatments may not effectively eliminate biofilms and can lead to side effects, such as antibiotic resistance and tolerance. We evaluated the impact of daily antibiotics or octanoic acid administration on *S. aureus* biofilm cells (from the 10-9 isolate). Following biofilm maturation, the treatments were administered once daily for four days, as illustrated in Fig. 5A. Daily administration of antibiotics had a limited impact on cell viability, with a maximum of 2-log reduction in CFU observed on day 4 for all antibiotic-treated groups. In contrast, daily administration of octanoic acid (25 mM) gradually reduced cell viability until the biofilm was completely eradicated at day 4 (Fig. 5B). Furthermore, continuous exposure to antibiotics increased the SCVs in biofilms. In particular, tetracycline treatment led to about 55% SCVs in the culture. In contrast, continuous exposure to octanoic acid did not lead to detectable SCV development (Fig. 5C and D). SEM images showed abnormal cell division in the antibiotic-treated groups (Fig. 5E) and the appearance of 'fried egg'-shaped SCVs [23]. In contrast, normal cell morphologies were observed in the untreated control and octanoic acid-treated biofilms (Fig. 4B). A study shows that the biofilm cell populations consisted of wild-type cells and SCVs, with the SCV subpopulation growing over time [29]. We also found that SCVs occur spontaneously in biofilms after 72 h of incubation without changing medium (Fig. S4), but the normal phenotype was restored after changing the medium (Fig. S5C and D, growth control). Next, we evaluated the effects of octanoic acid and antibiotic exposure on aged (72 h) biofilms. As shown in Fig. S5C and D, the SCV subpopulations were noticeably more prevalent in the antibiotic-treated groups than the control group. SCVs were particularly evident in the tetracycline-treated group (~75% SCVs). In contrast, the octanoic acid-treated biofilms were largely eradicated (Fig. S5B) and showed low prevalence of induced SCV phenotypes (Fig. S5C and D). These results demonstrate that octanoic acid can eradicate biofilms and carries a lower risk of SCV induction than antibiotics.

3.5. Octanoic acid attenuates expression of tetracycline-induced virulence factor genes in S. aureus biofilm cells

Previous studies have shown that the *S. aureus* cells surviving antibiotic treatments have a persister phenotype, which is characterized by low metabolic activity and elevated expression of stress response genes [5]. In *S. aureus*, the staphylococcus accessory regulator (Sar) and accessory gene regulator (Agr) signaling cascades regulate the expression of many virulence and biofilm formation factors via oligopeptide signaling molecules [30–32]. Examples include the *hla* gene encoding α-hemolysin, a toxic protein that can damage host tissues [32]. Fibronectin-binding protein A (FnbA) and clumping factor A (clfA) are adhesion proteins that allow the pathogen to interact with bovine mammary epithelial cells and contribute to biofilm formation [33–35]. Meanwhile, SigB controls stress response and survival genes, and it regulates biofilm formation through the SigB regulon, which is crucial for the development of chronic infections [36]. ArlR is a component of the ArlRS signal transduction system that controls virulence factors and antibiotic tolerance by directly controlling downstream gene expression. This action allows *S. aureus* to adapt and survive in various environments [37]. Rbf controls the expression of genes involved in biofilm formation and also affects pathogenesis-related gene expression [38]. In our experiments, we tracked expression of these genes to indicate virulence and biofilm formation ability of the cells.

Since biofilms that form after tetracycline treatment carry high proportions of SCVs with the persister phenotype, we investigated the effects of octanoic acid and tetracycline on the expression of virulence- and biofilm-related genes in biofilm cells. Treatment of the *S. aureus* isolate 10-9 biofilm with tetracycline dramatically induced the expression of *fnbA*, *agrA*, *rbf*, *arlR*, *hla* and *sigB* genes after 8 h and 24 h. In contrast, octanoic acid treatment did not upregulate expression of these genes. Notably, when tetracycline and octanoic acid were administered in a combined treatment, the tetracycline-induced gene expression pattern was attenuated (Fig. 6B).

3.6. Octanoic acid quickly kills persister cells and antibiotic-tolerant cells

Since SCV development is associated with persister cells, decreased antibiotic sensitivity and chronic infections [39], we next examined the bactericidal effects of octanoic acid and antibiotics on the surviving *S. aureus* cells in biofilms treated with antibiotics (Fig. 7A). For planktonic stage, *S. aureus* ATCC 12600 and isolate 10-9 cells (Fig. 7B), the MDK99 (minimum duration to kill 99% of bacteria) of octanoic acid and antibiotics were respectively within 2 and 8 h, and the respective MDK99.99 values were within 4 and 24 h. However, cells at the biofilm stage had an MDK99.99 of more than 24 h in all antibiotic-treated groups (*S. aureus* ATCC 12600, Fig. 7C), implying that persister cells were present in the biofilm stage. Moreover, when *S. aureus* biofilms were treated with cloxacillin (Fig. 7D) or tetracycline (Fig. 7E), the MDK99 values were respectively more than 8 and 24 h in all antibiotic-treated groups. Thus, antibiotic tolerance appeared to be increased in biofilm cells upon antibiotic treatment. In contrast, octanoic acid exhibited MDK99.99 values within 4 h, whether the *S. aureus* were at a planktonic stage (Fig. 7B) or biofilm stage (Fig. 7C). Octanoic acid also had MDK99.99 values less than 4 h for biofilms treated with cloxacillin (Fig. 7D) or tetracycline (Fig. 7E). These results showed that octanoic acid exhibits rapid bactericidal activity toward persister cells and antibiotic-tolerant cells in *S. aureus* biofilms.

4. Discussion

Chronic biofilm infections are notoriously difficult to cure with standard antibiotic treatments, and no effective treatment strategy has yet been developed for clearing biofilms in bovine mastitis. In this work, we demonstrate that octanoic acid does not suffer from the main

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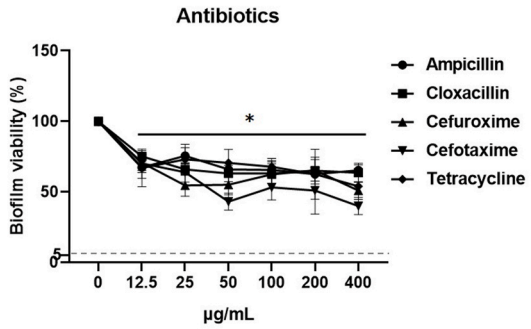

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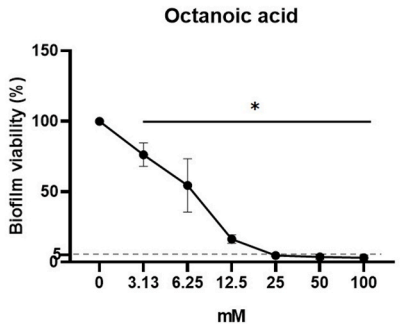

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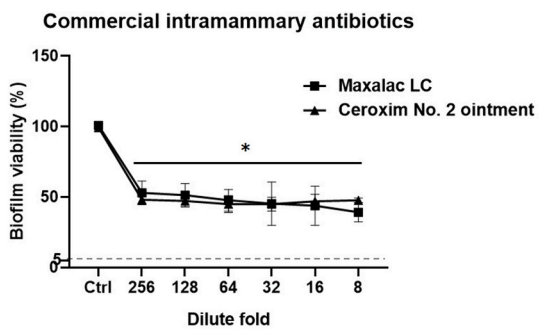
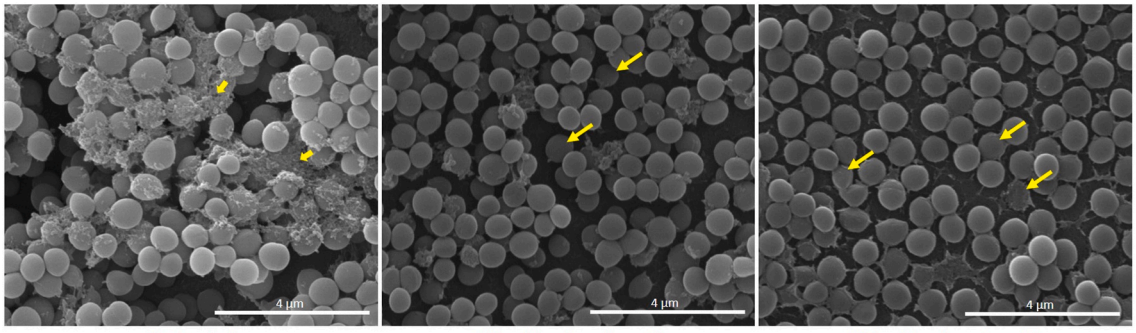


D

Antibiotic classes	Name	MBC	95% BEC	95% BEC/MBC (Fold)
	Octanoic acid	25-50 mM	25 mM	1x
Penicillin	Ampicillin	0.78 μg/mL	>400 μg/mL	>100
	Cloxacillin	0.39 μg/mL	>400 μg/mL	>100
Cephalosporin	Cefuroxime	3.13 μg/mL	>400 μg/mL	>100
	Cefotaxime	3.13 μg/mL	>400 μg/mL	>100
Tetracycline	Tetracycline	-	>400 μg/mL	-

MBC: minimum bactericidal concentration.
95% BEC: 95% biofilm eradication concentration.
-: undetermined.

E



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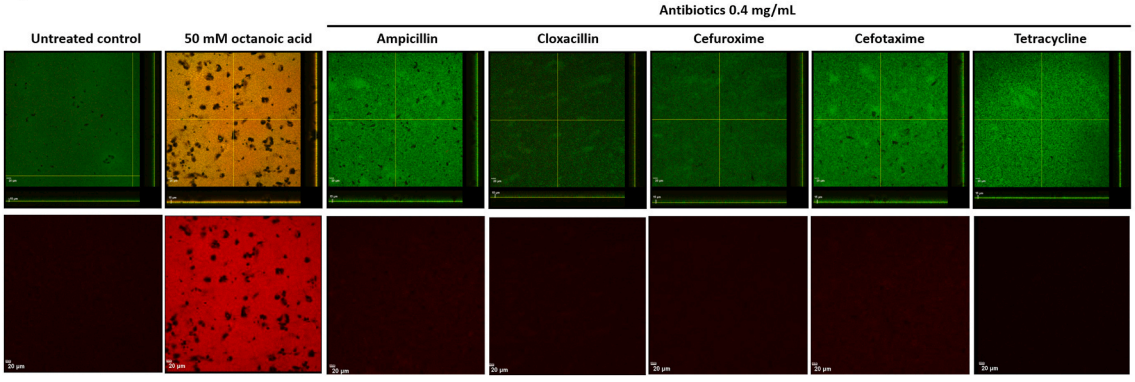

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Fig. 4. Evaluation of octanoic acid and antibiotics in *S. aureus* biofilm eradication assay. (A) *S. aureus* ATCC 12600 mature biofilm treated with antibiotics (0–400 μg/mL), (B) commercial intramammary antibiotics (diluted 1:8 to 1:256 in sBHI), and (C) octanoic acid (0–100 mM) for 24 h. Biofilm cell viability was quantified by Alamar Blue assay. Untreated control was taken as 100% cell viability. The dotted line represents 95% biofilm eradication. Octanoic acid and antibiotic 95% biofilm eradication concentration (95% BEC) and 95% BEC/MBC are listed in table (D). All values represent the mean ± SD of three individual experiments. $^{*}p < 0.05$ compared with the untreated control. (E) SEM images at 30,000 × magnification in ATCC 12600 *S. aureus* mature biofilm treatment by octanoic acid (untreated, 25 and 50 mM). Yellow arrows represent the ECM of *S. aureus* mature biofilm. Yellow thin arrows represent collapsed cells in octanoic acid-treated biofilms. Scale bars = 4 μm. (F) ATCC 12600 *S. aureus* mature biofilm cell viabilities were analyzed after culture in sBHI (Untreated), 50 mM octanoic acid, or 0.4 mg/mL antibiotics by CLSM at 20 × magnification. BacLight LIVE/DEAD viability kit was used to stain biofilm cells. Bacteria with intact cell membranes were stained fluorescent green (SYTO™ 9), while bacteria with damaged membranes were stained fluorescent red (propidium iodide). CLSM imaging shows green and red fluorescence (upper), representing the total cell content in biofilms, and red fluorescence alone (lower), representing the content of dead cells in biofilms. Lateral white scale bars = 20 μm. Longitudinal white scale bar = 15 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

drawbacks of antibiotic therapy for bovine mastitis, as it can prevent formation and eradicate established *S. aureus* biofilms. When treating biofilms, octanoic acid is less likely than antibiotics to induce SCVs. It can also quickly kill biofilm cells and antibiotic-tolerant cells. These advantages of octanoic acid are expected to prevent disease progression to chronic infection.

Antibiotic therapy is currently the primary strategy for treating bovine mastitis, but it suffers from low cure rates and leaves antibiotic residuals in raw milk [40]. Moreover, recent regulations encourage the reduced usage of antibiotics or their replacement with alternative therapies for dairy cows. The European Medicines Agency (EMA) has categorized available antibiotic substances into three categories, with veterinary drugs restricted to the first two categories [3]. Extensive efforts have recently been made to develop new strategies for treating bovine mastitis, including the use of probiotics, bacteriocins, bacteriophages, phage endolysins, lysostaphin and cytokines; however, commercially available agents with efficacy for eliminating MCPs are still lacking, and some available agents cannot be used with raw milk [1, 41–44]. Another approach has been to develop mastitis vaccines against major targets of *S. aureus*, *Streptococcus uberis,* and *E. coli*. While some commercial vaccines are now available, vaccination is costly and generally ineffective against bovine mastitis because various pathogens can cause the condition [45].

Octanoic acid is a saturated medium-chain fatty acid with antimicrobial and anti-inflammatory properties that is found naturally in milk. The antibacterial and anti-biofilm activity of octanoic acid has been published in several studies [11–17]. Our study extends previous findings by focusing on using octanoic acid to prevent pathological progression to chronic infection. Bovine mastitis can be caused by multiple pathogens, making it challenging to select an effective antibiotic. Although broad-spectrum antibiotics are available, the drugs may not be effective on all pathogens. Thus, proper antibiotic selection requires first identifying the pathogen responsible for the infection. This procedure can be challenging, especially in dairy farming operations. In addition, antibiotic selection guided by culture-based identification is not always effective in clinical cases, as there may be a mismatch between *in vitro* sensitivity results and practical outcomes [44]. For instance, *S. aureus* mastitis should be susceptible to antibiotics according to *in vitro* data. However, adaptation of the pathogen to the host environment and the host response to infection can render antibiotics ineffective, as with abscesses and biofilm formation [46]. Octanoic acid shows broad-spectrum microbicidal activity against various infectious pathogens in milk. Administering octanoic acid during the early infection stage in cows can potentially shorten the pathogen identification process and minimize the risk of infection transmission within the dairy farm.

In previous studies, recurring mastitis was found to be caused by the same pathogenic species as seen in prior infections for 32.6% of cases [47], and *S. aureus* was shown to be a prominent cause of bovine mastitis that frequently results in long-term, chronic and recurring infections [48–50]. Here, we found that almost all strains with biofilm-forming capacity were isolated from recurrent mastitis cases (udders No. 8–13). However, these strains were still sensitive to the antibiotics used to treat the infections (ampicillin and cloxacillin). This result implies that the isolated pathogen can somehow evade or tolerate antibiotic treatments to which it is sensitive; such an outcome may be related to biofilm-associated tolerance or persister cells.

High concentrations of antibiotics cannot eradicate biofilms [51], even after repeated treatment. In our study, antibiotic resistance in surviving isolates was minimal, and the isolates instead exhibited SCV phenotypes. Continuous exposure to antibiotics enhances the SCV subpopulation in biofilm cells and suppresses bacterial proliferation during treatment, which should reduce immune response and somatic cell count in the milk. However, stopping antibiotics would allow the SCV bacteria to regrow, resulting in recurrence of infection. In contrast, octanoic acid effectively eradicates biofilms and does not readily induce SCVs. As such, octanoic acid therapy is unlikely to promote biofilm formation. The treatment also rapidly kills pathogenic cells, even those in biofilms or antibiotic-tolerant cells. In summary, our data suggest that octanoic acid treatment is a strong candidate for preventing progression to chronic mastitis, as it is effective against various MCPs at planktonic and biofilm stages. Thus, treatment with octanoic acid may be a new effective strategy for controlling intractable biofilm-related infections, such as bovine mastitits.

5. Conclusion

Antibiotics are the primary treatment for bovine mastitis, although the cure rate is poor and the strategy has several drawbacks. When a dairy cow is found to be infected, it is most commonly treated immediately with a broad spectrum of intramammary antibiotics in order to prevent pathogen spread. During the treatment and withdrawal periods, raw milk produced by the cow must be discarded due to antibiotic residues, which causes major economic losses. Octanoic acid is a Generally Recognized as Safe (GRAS) compound, and this study demonstrates that octanoic acid exerts broad-spectrum bactericidal activity toward mastitis-causing pathogens in milk, effectively preventing *S. aureus* biofilm formation. More importantly, our data show that octanoic acid can eradicate biofilms and quickly kill antibiotic-tolerant pathogens. Overall, this work demonstrates that octanoic acid is a powerful antimicrobial and potential treatment for different stages of bovine mastitis pathogenesis. In this study, we focused on the major biofilm-forming pathogen *S. aureus*, testing ATCC 12600 and clinical isolate 10-9 as representative models. However, the *in vivo* cattle environment may be highly complex and include other pathogens that support biofilm formation. Thus, further investigations will be needed to thoroughly test the applicability of octanoic acid for treatment of bovine mastitis.

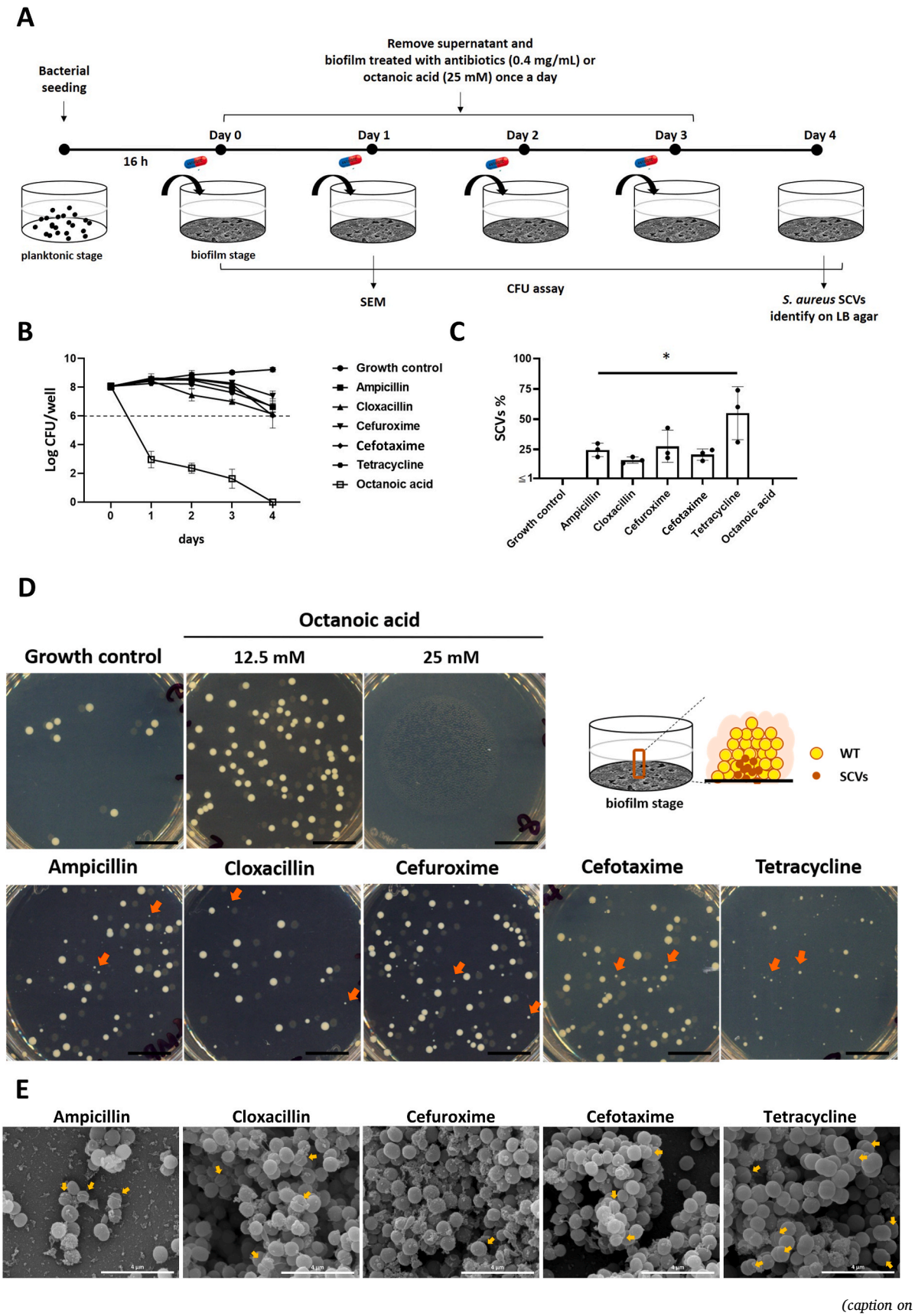

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Fig. 5. Antibiotic treatment induces SCV development in biofilms formed by *S. aureus* isolated from recurrent bovine mastitis. (A) Schematic diagram of the experimental process. Day 0 marks the day of mature biofilm formation. Mature biofilms of *S. aureus* isolate 10-9 were treated with 25 mM octanoic acid or 0.4 mg/mL antibiotic. Fresh drugs and sBHI (biofilm medium) were changed once a day until the third day. (B) Daily measurements of biofilm cell viability by CFU assay. The dotted line represents a decrease in cell viability by 2 log CFU compared to day 0. (C) Quantification of SCVs ratios (SCVs among total live cells) in different treatment groups (less than 1% is not detectable). SCVs were identified by LB agar plate method on the fourth day after drug treatment; SCVs were defined as colonies at least 10-fold smaller than normal colonies (growth control) [23]. All values represent the mean ± SD of three individual experiments. *$p < 0.05$ compared with the growth control. (D) The images of colony morphotypes on LB agar for each group (day 4). Scale bars = 1 cm. The orange arrows indicate SCV phenotypes developed upon exposure to antibiotics. (E) SEM analysis of morphological changes in biofilm cells after antibiotic treatment (day 1). The yellow arrows indicate SCV 'fried egg' phenotypes [23] in the antibiotic treatment groups (compared with untreated control biofilm, see Fig. 4B). Images are magnified 30,000 × ; scale bars = 4 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

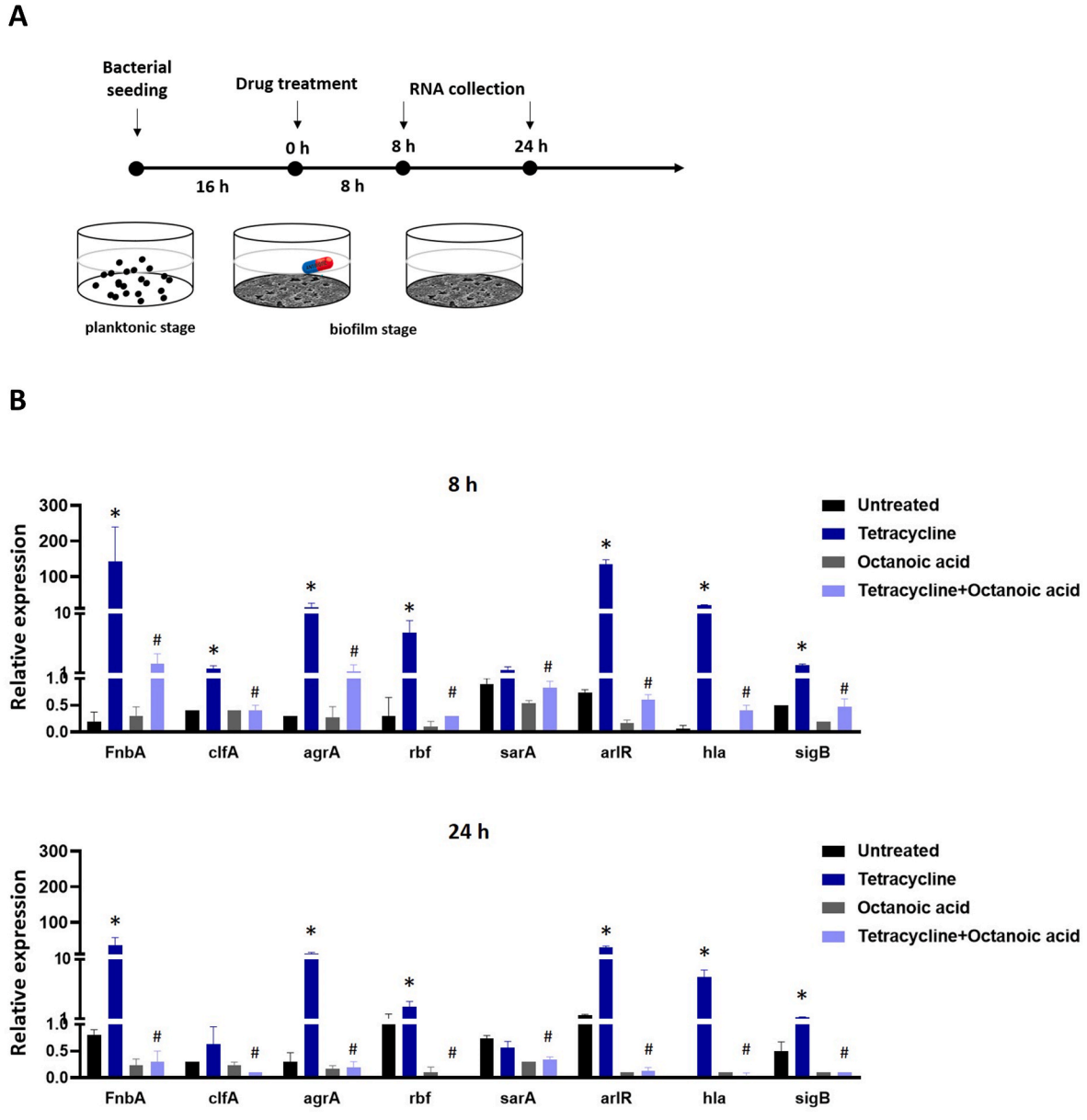

Fig. 6. Comparative transcript levels of virulence genes in *S. aureus* biofilms after treatment with octanoic acid, tetracycline, and octanoic acid/tetracycline combination. (A) Schematic diagram of the experimental procedure. (B) Mature biofilms of *S. aureus* isolate 10-9 were treated with 4 mM octanoic acid, 0.4 mg/mL tetracycline or a combination of both. After 8 h and 24 h incubation, biofilm cells were harvested for RNA extraction. Quantification of virulence gene (*fnbA, clfA, agrA, rbf, sarA, arlR, hla* and *sigB*) transcription in different treatment groups. Data are expressed as fold-change compared to 0 h (untreated). All values represent the mean ± SD of three individual experiments. *$p < 0.05$ compared with the untreated group (8 h and 24 h). #$p < 0.05$ compared with the tetracycline group (8 h and 24 h).

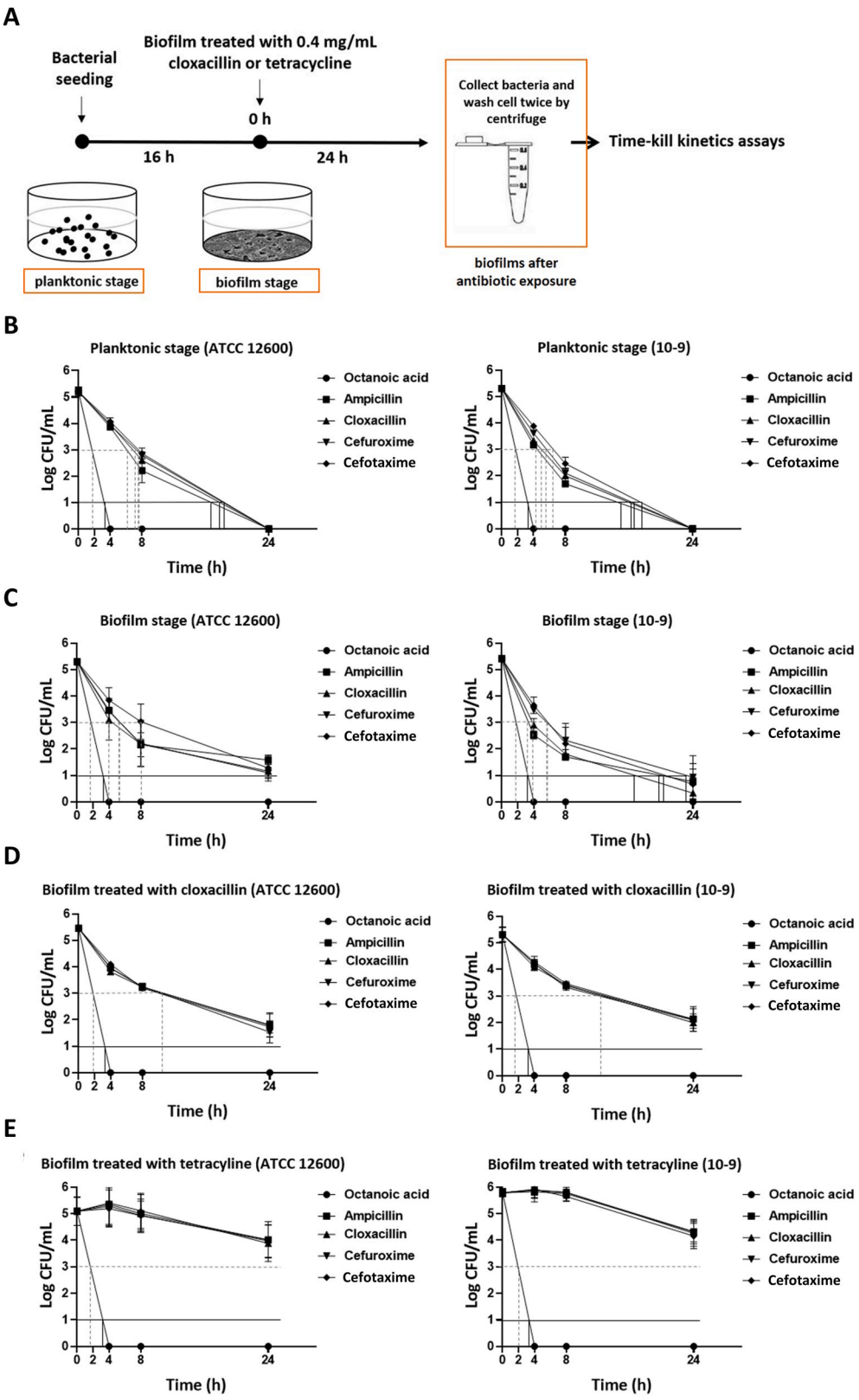

Fig. 7. Comparative time-kill curves of octanoic acid and antibiotics against *S. aureus* cells at planktonic stage, biofilm stage, or biofilms after antibiotic (tetracycline or cloxacillin) exposure. (A) Schematic diagram of the experimental design. Representative time-kill curves for *S. aureus* ATCC 12600 and isolate 10-9 after exposure to octanoic acid at 2 × MBC (100 mM) and antibiotics at 4 × MBC (ampicillin, cloxacillin, cefuroxime and cefotaxime were respectively treated at 3.125, 1.56, 12.5, and 12.5 μg/mL for *S. aureus* ATCC 12600 and 1.56, 0.39, 3.13, and 6.25 μg/mL for *S. aureus* 10-9). *S. aureus* cells collected from the planktonic stage (B), biofilm stage (C), or biofilms after tetracycline (D) or cloxacillin (E) exposure. CFU/mL of each group was calculated at 0, 4, 8, and 24 h. The dotted line represents the minimum duration to kill 99% of the population (MDK99). The solid line represents the minimum duration to kill 99.99% of the population (MDK99.99). All values represent the mean ± SD of three individual experiments.

Nevertheless, our study enhances understanding of biofilm-related factors and may facilitate the development of targeted strategies for effective mastitis control and treatment.

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CRediT authorship contribution statement

Wen-Chun Lin: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Kai-Chen Hsu:** Investigation, Data curation, Methodology, Formal analysis. **Ming-Feng You:** Data curation, Methodology, Formal analysis. **Kuo-Hua Lee:** Conceptualization, Data curation. **Chau-Hwa Chi:** Conceptualization, Supervision. **Jyh-Yih Chen:** Conceptualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2023.100149.

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