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Inhibitory effect of propionate on *Staphylococcus aureus* growth and infection

Propionate에 의한 황색포도상구균 성장 억제 및 감염 제어

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ABSTRACT

Inhibitory effect of propionate on *Staphylococcus aureus* growth and infection

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Objectives

Staphylococcus aureus is a Gram-positive pathogen that can cause various diseases including skin and soft tissue infections, pneumonia, endocarditis, and sepsis. Moreover, *S. aureus* is adept at acquiring antibiotic resistance. Methicillin-resistant *S. aureus* (MRSA) is a serious threat in healthcare settings and in the communities. Infections by vancomycin-resistant *S. aureus* and multidrug-resistant *S. aureus* have also been increasing. Therefore, a novel strategy to combat antibiotic-resistant *S. aureus* infections is needed. Short-chain fatty acids (SCFAs) are metabolites produced by gut microbiota by fermentation of dietary fibers and non-digestible carbohydrates. The major SCFAs in the gut are acetate, butyrate, and propionate. Apart from their immunomodulatory roles in the host, SCFAs have been suggested to have antimicrobial effects on some pathogenic bacteria such as *Helicobacter*

pylori and *Salmonella enterica*. However, the effects of SCFAs on *S. aureus* have not been extensively studied. The aim of this study was to investigate the effects of SCFAs, acetate, propionate or butyrate, on *S. aureus* growth and infection.

Methods

MRSA USA300 was cultured in the presence or absence of various doses of SCFAs, acetate, propionate, or butyrate. Optical density at 600 nm was measured to examine bacterial growth. The effects of SCFAs on multidrug-resistant clinical isolates were also investigated. The minimum inhibitory concentration/minimum bactericidal concentration test was conducted to determine if SCFAs had bacteriostatic or bactericidal effects. Bacterial morphology was observed under the scanning electron microscope. To determine the effects of SCFAs on MRSA skin infection, mice were subcutaneously infected with MRSA, with or without acetate, propionate, or butyrate. Three days post-infection, abscess size and weight were measured, and homogenates of abscesses were used to measure the bacterial load. The expression of interleukin (IL)-1β, a major cytokine in skin infection, and IL-6, a proinflammatory cytokine, in homogenates was measured by enzyme-linked immunosorbent assay. Abscesses were cryosectioned and subjected to histological analysis by hematoxylin and eosin staining and Gram staining. The effect of propionate treated after skin infection was initiated was also investigated. To determine the role of cell wall components in the growth inhibition, growth studies were conducted with *S. aureus* deficient of lipoteichoic acid (Δ*ltaS*), wall teichoic acid (Δ*tagO*), lipoprotein (Δ*lgt*), or $_{D}$-alanylation of teichoic acids (Δ*dltA*). A $_{D}$-alanylation inhibitor of teichoic acids, amsacrine, was used for MRSA. Amsacrine and propionate were co-treated *in vitro* and *in vivo*. To study the action mechanism at the molecular level, the Nebraska Transposon Mutant Library of MRSA USA300 was used for growth studies. The effect of propionate on other Gram-positive bacteria was also investigated.

Results

Of the three SCFAs, propionate most potently inhibited the growth of MRSA USA300, inhibiting its growth in a dose-dependent manner. Butyrate had some inhibitory effects, while acetate had minimal effects. Propionate had a bacteriostatic effect, only inhibiting bacterial growth without killing it, and did not cause physical damage. Propionate inhibited the growth of multidrug-resistant clinically isolated strains. Propionate ameliorated MRSA skin infection, by lowering abscess formation and bacterial load, without having toxic effects in mice at the concentration used. Excessive cytokine expression, namely IL-1β and IL-6, also decreased. Propionate also reduced abscess formation when treated after MRSA infection was initiated. *S. aureus* deficient of lipoteichoic acids or wall teichoic acids was more susceptible to propionate than the wild-type. In addition, *S. aureus* deficient of D-alanine motifs common in teichoic acids was more susceptible to propionate. Concordantly, MRSA treated with amsacrine, which inhibits D-alanylation of teichoic acids, was more susceptible to propionate. Co-treatment of amsacrine and propionate further ameliorated MRSA skin infection. Both propionate and amsacrine were not toxic at the concentrations used. MRSA deficient of a gluconeogenesis enzyme or tricarboxylic acid cycle enzyme was more susceptible to propionate, while MRSA deficient of a glycolytic enzyme was not, suggesting that propionate may affect bacterial metabolism. The growth of *Streptococcus pneumoniae* or *Enterococcus faecium* was potently inhibited, while that of *Staphylococcus epidermidis* or *Enterococcus faecalis* was moderately inhibited by propionate. The growth of *Streptococcus gordonii* or *Lactobacillus plantarum* was not inhibited by propionate.

Conclusions

Collectively, propionate inhibited the growth of *S. aureus*, including clinically isolated multidrug-resistant *S. aureus*, and decreased the pathology of MRSA skin infection. Since propionate did not have toxic effects *in vivo*, and is a metabolite present in our body, it is likely to have fewer or no side effects in the host compared with other antibiotics. Moreover, as co-treatment of propionate and a D-alanylation

inhibitor, both of which were not toxic in the concentrations used, further reduced pathology, a combination therapy may be an alternative strategy to treat multidrug-resistant *S. aureus* infections.

Keywords: *Staphylococcus aureus*, MRSA, Propionate, Short-chain fatty acids, $_{D}$-Alanine

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CONTENTS

Chapter I. Introduction

Staphylococcus aureus is a Gram-positive bacterium that frequently colonizes humans. It is a major pathogen that causes various diseases including skin and soft tissue infections (SSTIs), pneumonia, and endocarditis, and is the most frequent bacterium associated with sepsis [1, 2]. SSTIs are the most common forms of *S. aureus* infections and can lead to the spread of *S. aureus* to other parts of the body, often resulting in serious diseases such as bacteremia or pneumonia [3]. Moreover, *S. aureus* is the most common cause of SSTIs among patients in the emergency department [4]. In *S. aureus* infections, neutrophils are first recruited to the site of infection, where they produce interleukin (IL)-1β, a cytokine which plays an important role in immunity against *S. aureus* infection, but which can also cause immunopathology [5, 6]. In addition, *S. aureus* is able to evade the host's immune system. It can resist phagocytosis by neutrophils, and resist killing by antimicrobial peptides or lysozyme via various mechanisms [7-9]. Furthermore, *S. aureus* is able to resist killing inside neutrophils and survive inside phagosomes [10]. It has been suggested that *S. aureus* can take advantage of these cells by residing in them to contribute to infection, and disseminate to other parts of the body such as the bone or heart [11].

Since there is no vaccine yet available to prevent *S. aureus* infections, treatment of *S. aureus* infections mostly relies on antibiotics [12]. However, *S. aureus* is adept at acquiring antibiotic resistance. Methicillin-resistant *S. aureus* (MRSA) is a serious threat in not only healthcare settings but also in the communities, which is predicted to kill approximately 19,000 patients per year in the US alone, which is similar to

the number of deaths by AIDS, tuberculosis, and hepatitis combined [13, 14]. The worldwide prevalence of MRSA is increasing, and interestingly, South Korea has a particularly high MRSA prevalence, with 73% of clinical isolates being methicillin-resistant [15, 16]. SSTIs represent approximately 90% of total infections caused by community-acquired MRSA [3]. However, resistance against topical antimicrobials, such as fusidic acid, bacitracin, neomycin, or mupirocin, used against *S. aureus* skin infections, has emerged [17-19]. Moreover, infections by vancomycin-resistant *S. aureus* and multidrug-resistant *S. aureus* have been increasing [20, 21]. In addition, emerging resistance to newer classes of antibiotics such as linezolid, and side effects of antibiotics are emphasizing the limited treatment options [22-24]. Therefore, a novel strategy to combat antibiotic-resistant *S. aureus* infections is needed. Recently, combination therapy and the concept of synthetic lethality have been gaining attention as strategies to overcome antibiotic-resistant *S. aureus* infections [25, 26]. Combination therapy has been thought to be more effective and less prone to resistance, and has been employed for MRSA infections [27].

Short-chain fatty acids (SCFAs) are metabolites produced by gut microbiota when dietary fibers and non-digestible carbohydrates are fermented in the colon [28]. The major SCFAs in humans, acetate, propionate, and butyrate, represent more than 95% of total SCFAs, and are produced in a molar ratio of approximately 60:20:20 [29]. The concentration of SCFAs is in the range of 70 mM to 140 mM where they are the most abundant [30]. SCFAs are produced by many different commensal bacteria via diverse pathways. The Bacteroidetes phylum mainly produces acetate and propionate, whereas the Firmicutes phylum mainly produces butyrate [31]. SCFAs

drain via the portal vein and reach the periphery, including the lung, liver, or bone marrow to exert beneficial metabolic effects [30, 32]. In addition, SCFAs are produced by commensals in other sites such as the skin as well [33]. SCFAs have various roles in the host, often simply acting as energy sources for colonocytes, and leading to enhanced mucus and antimicrobial peptides production [34]. Moreover, SCFAs regulate the immune system to maintain host immune homeostasis primarily in the gut, by inducing regulatory T cell development and helping maintain the immune tone [32, 35]. SCFAs can also induce effector T cells, such as Th17 cells, to promote immunity [36]. SCFAs and a high fiber diet, which results in an increased production of SCFAs, have been considered possible therapeutic modifications for inflammatory bowel disease, type 1 diabetes, allergy, and other inflammatory conditions [37-39].

Apart from their immunomodulatory roles in the host, SCFAs have antimicrobial effects on some pathogenic bacteria. The degree of toxicity mediated by SCFAs varies among different bacterial species. For example, butyrate inhibits the growth of *Helicobacter pylori* by exhibiting destructive effects on its cell envelope [40], and regulates the virulence of *Salmonella enterica* serovars Typhimurium and Enteritidis (*S.* Typhimurium and *S.* Enteritidis) [41]. Acetic acid inhibits the growth of *Escherichia coli* by interfering with methionine biosynthesis [42]. Propionate suppresses *S.* Typhimurium invasion and also inhibits its growth [43, 44]. Furthermore, it has been suggested that the fermentation products of *Propionibacterium acnes*, which contain various metabolites and proteins including propionic acid and butyric acid, inhibit *S. aureus* colonization [45]. Moreover,

propionic acid has been suggested to inhibit *S. aureus* growth by reducing bacterial intracellular pH [46]. However, although propionic acid can change the pH of the extracellular medium, the effects of the three SCFAs, acetate, propionate, or butyrate, which do not affect pH, have not been studied. In this study, the effects of SCFAs, which are biocompatible, on *S. aureus in vitro* and *in vivo* were studied, and an alternative combination treatment strategy to control antibiotic-resistant *S. aureus* infections was investigated.

Chapter II. Materials and Methods

2.1. Reagents and chemicals

SCFAs, sodium acetate, sodium propionate, and sodium butyrate, were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). SCFAs were dissolved in endotoxin-free distilled water (Dai Han Pharm Co. Ltd., Seoul, Korea), and filtered with a syringe filter (0.2 μm) purchased from Corning (Corning, NY, USA) prior to use. Luria Bertani (LB) broth was purchased from LPS solution (Daejeon, Korea). Trypticase soy broth (TSB), brain heart infusion (BHI), Todd-Hewitt (TH), yeast extract, and Bacto agar were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Lactobacilli deMan, Rogosa, and Sharpe (MRS) broth was purchased from Neogen (Langing, MI, USA). 2,2,2-Tribromoethanol and 2-methyl-2-butanol were purchased from Sigma-Aldrich Inc. Amsacrine (AMSA) was purchased from Abcam (Cambridge, UK). Hematoxylin and eosin were purchased from Sigma-Aldrich Inc. and BBC Biochemical (Mount Vernon, WA, USA), respectively. Crystal violet and safranin were purchased from Sigma-Aldrich Inc. Iodide solution was purchased from Samchun Chemicals (Seoul, Korea).

2.2. Bacterial strains and culture conditions

S. aureus strains used in this study are listed in Table 1. MRSA USA300 wild-type (WT) and mutants were provided by the Network on Antimicrobial Resistance in *S. aureus* (NARSA) for distribution by BEI Resources, NIAID, NIH: Nebraska Transposon Mutant Library Screening Array (NR-48501). Clinically-isolated *S.*

aureus and *Staphylococcus epidermidis* were obtained from the National Culture Collection for Pathogens (Osong, Korea). Strains and isolation sites in parentheses are as follows: MRSA NCCP 11485 (urine), 11486 (urine), 14565 (blood), 14566 (abscess), 14567 (abscess), 14568 (catheter tip), 14569 (abscess), 14748 (nose), 14750 (blood), 14751 (ear), 14769 (transtracheal aspirates), and vancomycin intermediate-resistant *S. aureus* (VISA) NCCP 13846 (pus), 13853 (pus), and 13863 (pus), and *S. epidermidis* NCCP 14768 (blood). These strains were cultured in TSB at 37°C with shaking. WT *S. aureus* RN4220 [47] and its lipoteichoic acid (LTA)-deficient (Δ*ltaS*) [48], wall teichoic acid (WTA)-deficient (Δ*tagO*) [49], lipoprotein (LPP)-deficient (Δ*lgt*) [50] mutants, and their complement strains of each (Δ*ltaS*/pM101-*ltaS* [48], Δ*tagO*/pS*tagO* [51], Δ*lgt*/pS*lgt* [50], and Δ*dltA*/p0793 [49]) were kindly provided by Prof. Bok-Luel Lee (Pusan University, Busan, Korea). WT, Δ*lgt*, Δ*lgt*/pS*lgt*, Δ*dltA*, and Δ*dltA*/p0793 *S. aureus* were cultured in LB broth at 37°C with shaking [49, 50]. Δ*ltaS*, Δ*ltaS*/pM101-*ltaS*, Δ*tagO*, and Δ*tagO*/pS*tagO S. aureus* were cultured in LB broth at 30°C with shaking as previously described [48, 49]. *Enterococcus faecalis* ATCC 29212 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). *Enterococcus faecium* KACC 11954 was obtained from Korean Agricultural Culture Collection (Wanju, Korea). Enterococci were cultured in BHI broth with shaking at 37°C. *Streptococcus gordonii* CHI, which was kindly provided by Dr. Paul M. Sullam (University of California at San Francisco, CA, USA), was cultured in TH broth supplemented with 0.5% yeast extract (THY) at 37°C. *Lactobacillus plantarum* KCTC 10887BP was obtained from Korean Collection for Type Cultures (Jeongeup, Korea), and was cultured in MRS broth at 37°C.

Table 1. *S. aureus* strains used in this study

Strain	Characteristics	Source or reference
USA300	MRSA	Nebraska Transposon Mutant Library
RN4220 WT	Lab strain	[47]
RN4220 Δ*ltaS*	Loss of LTA	[48]
RN4220 Δ*tagO*	Loss of WTA	[49]
RN4220 Δ*lgt*	Loss of lipoprotein lipid modification	[50]
RN4220 Δ*dltA*	Loss of D-ala modification in LTA and WTA	[49]
RN4220 Δ*ltaS*/pM101-*ltaS*	Δ*ltaS* strain containing a plasmid harboring the *ltaS* gene	[48]
RN4220 Δ*tagO*/p*StagO*	Δ*tagO* strain containing p*StagO* plamid	[51]
RN4220 Δ*lgt*/p*Slgt*	Δ*lgt* strain containing a plasmid harboring the *lgt* gene	[50]
RN4220 Δ*dltA*/p0793	Δ*dltA* strain containing plasmid harboring intact *dltABCD*	[49]
NCCP 11485	Urine; resistance to methicillin, oxacillin, penicillin, erythromycin, cefazolin, amoxicillin/clavulanic acid, gentamicin, tetracycline, mupirocin	National Culture Collection for Pathogens
NCCP 11486	Urine; resistance to methicillin, oxacillin, penicillin, erythromycin, clindamycin, cefazolin, amoxicillin/clavulanic acid, ofloxacin, tetracycline, rifampin	National Culture Collection for Pathogens
NCCP 14565	Blood; resistance to methicillin, penicillin, tetracycline, linezolid, quinupristin/dalfopristin	National Culture Collection for Pathogens
NCCP 14566	Abscess; resistance to methicillin, penicillin, cefoxitin	National Culture Collection for Pathogens
NCCP 14567	Abscess, resistance to methicillin, penicillin, cefoxitin, erythromycin, clindamycin, tetracycline	National Culture Collection for Pathogens
NCCP 14568	Catheter tip; resistance to methicillin, penicillin, cefoxitin, ofloxacin, erythromycin, clindamycin, tetracycline	National Culture Collection for Pathogens
NCCP 14569	Abscess; resistance to methicillin, penicillin, cefoxitin, erythromycin	National Culture Collection for Pathogens
NCCP 14748	Nose; resistance to methicillin, penicillin, cefoxitin, erythromycin	National Culture Collection for Pathogens
NCCP 14750	Blood; resistance to methicillin, penicillin, cefoxitin, erythromycin	National Culture Collection for Pathogens
NCCP 14751	Ear; resistance to methicillin, penicillin, cefoxitin, ofloxacin, erythromycin, clindamycin, tetracycline	National Culture Collection for Pathogens
NCCP 14769	Transtracheal aspirates; resistance to methicillin	National Culture Collection for Pathogens
NCCP 13846	Pus; intermediate resistance to vancomycin, resistance to methicillin, oxacillin, penicillin, erythromycin	National Culture Collection for Pathogens
NCCP 13853	Pus; intermediate resistance to vancomycin, resistance to methicillin, oxacillin, penicillin, erythromycin, clindamycin, amoxicillin/clavulanic acid, ofloxacin, gentamicin, tetracycline	National Culture Collection for Pathogens
NCCP 13863	Pus; intermediate resistance to vancomycin, resistance to methicillin, oxacillin, penicillin, erythromycin, amoxicillin/clavulanic acid, gentamicin, tetracycline	National Culture Collection for Pathogens

2.3. Effects of SCFAs or AMSA on bacterial growth *in vitro*

The general experimental scheme is illustrated in Fig. 1. A single colony of bacteria was inoculated and cultured overnight. One percent of an overnight culture was inoculated to fresh medium, in the presence or absence of various doses of SCFAs (1.56, 3.13, 6.25, 12.5, 25, 50, or 100 mM) and/or AMSA (1.56, 3.13, 6.25, 10, 12.5, 20, or 25 μg/ml) in flat bottom, non-coated polystyrene 96-well plates (Thermo Scientific, Waltham, MA, USA). Bacteria were cultured at 30 or 37°C accordingly with or without shaking and optical density at 600 nm was measured using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). To confirm that propionate does not affect the pH, the pH of the extracellular medium was measured throughout the growth experiments using pH indicator strips (Whatman pH indicators, GE Healthcare, Chicago, IL, USA). To determine synergy, the fractional inhibitory concentration index (FICI) was calculated using the checkerboard assay as previously described [52, 53] with propionate and AMSA. The FICI was interpreted as FICI $\leq$ 0.5 synergy, 0.5 < FICI $\leq$ 1 partial synergy, 1 < FICI $\leq$ 4 additive effect or indifference, and 4 < FICI antagonism [52, 53].

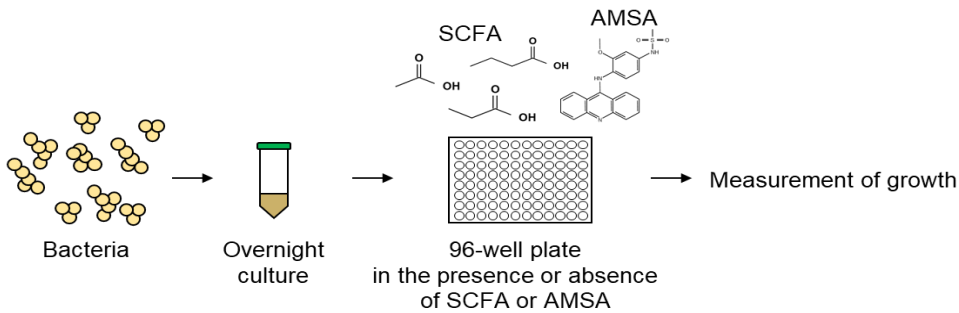

Figure 1. Experimental scheme of *in vitro* bacterial growth experiments. A single colony of bacteria was cultured overnight in media. Then, bacteria were cultured in various doses of SCFAs and/or AMSA. Growth was measured by reading optical density values at 600 nm.

2.4. Minimum inhibitory concentration/minimum bactericidal concentration (MIC/MBC) test

The MIC/MBC test was conducted using the microdilution method adopted from the Clinical and Laboratory Standards Institute (CLSI) guidelines [54] with minor modifications to determine if the agents used are bacteriostatic or bactericidal. Bacteria at 5×10^5 CFU/ml were inoculated in media containing serially diluted antimicrobial substances (0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000, 2000, or 3000 mM SCFAs, with 10 μg/ml AMSA where indicated, or 1.56, 3.13, 6.25, 12.5, 25, 50, 100, or 200 μg/ml AMSA), and cultured for 24 h. The MIC was defined as the minimum concentration that resulted in no visible growth after 24 h. Optical density at 600 nm was measured to confirm. To determine the MBC, wells that did not result in bacterial growth were inoculated in fresh media, free of antimicrobial substances. Optical density at 600 nm was measured after an additional 24 h. The MBC was defined as the minimum concentration that resulted in no growth even after inoculation in fresh media.

2.5. Scanning electron microscopy

S. aureus was cultured to mid-log phase, then the optical density at 600 nm was set to 0.5. *S. aureus* was cultured in the presence or absence of 50 mM propionate overnight in round-bottom 24-well plates (Thermo Scientific, Waltham., MA, USA) at 37°C without shaking. The supernatants were removed, and the cells were washed with phosphate-buffered saline (PBS). Bacteria were fixed with PBS containing 2% paraformaldehyde and 2.5% glutaraldehyde after they were first rinsed with the

solution. The samples were washed with PBS, then fixed with 1% osmium tetroxide for 1.5 h. Samples were washed with distilled water, and dehydrated through an ethanol series: 70, 80, 90, and 95% for 15 min each, then 100% for 15 min three times. Then, samples were dried using a critical point dryer (HCP-2, Hitachi, Tokyo, Japan), and sputter-coated with platinum using an ion sputter (Quorum Q150T S, Quorum Technologies Ltd., East Grinstead, UK). Samples were observed using a scanning electron microscope (S-4700, Hitachi, Tokyo, Japan) at 15 kV.

2.6. MRSA skin infection

All animal experiments were conducted under the approval of Institutional Animal Care and Use Committee of Seoul National University (SNU-170518-5 and SNU-181002-2). Eight- to ten-week-old female C57BL/6 mice were purchased from Orient Bio (Seongnam, Korea). Animals were housed in specific pathogen-free conditions under a 12 h light-dark cycle. A *S. aureus* skin infection model was used as previously described [55], with slight modifications. The experimental scheme is shown in Fig. 2. MRSA was cultured to mid-log phase, washed, and resuspended in endotoxin-free distilled water alone, or in endotoxin-free distilled water containing 50 mM SCFA and/or 10 μg/ml AMSA, to a final concentration of 3×10^7 CFU/ml. Control samples containing SCFA and/or AMSA alone were also prepared. Mice were anesthetized with a mixture of 2,2,2-tribromoethanol and 2-methyl-2-butanol. The flank area of mice was shaved with an electric hair clipper and depilatory cream. After disinfecting the injection sites with ethanol, mice were challenged subcutaneously with 3×10^6 CFU MRSA in a volume of 100 μl alone, or with SCFA

and/or AMSA. Animals were monitored daily for three days. For treatment experiments, mice were subcutaneously infected with 3×10^6 CFU MRSA in a volume of 100 µl. Propionate was injected 1, 24, and 48 h post-infection (h.p.i.) via intra-abscess injection [56]. On day 3, after euthanasia, abscess length and width were measured with a digital caliper (Mitutoyo Corporation, Kawasaki, Japan). Abscesses were aseptically excised, weighed, and homogenized. Homogenates were serially diluted and plated on TSB agar plates to measure bacterial load. To confirm that the effect of propionate on host cells is minimal, mice were subcutaneously injected with 1×10^8 or 1×10^9 CFU of heat-killed MRSA alone or with 50 mM propionate. On day 3, after euthanasia of mice, the abscess size and weight were measured.

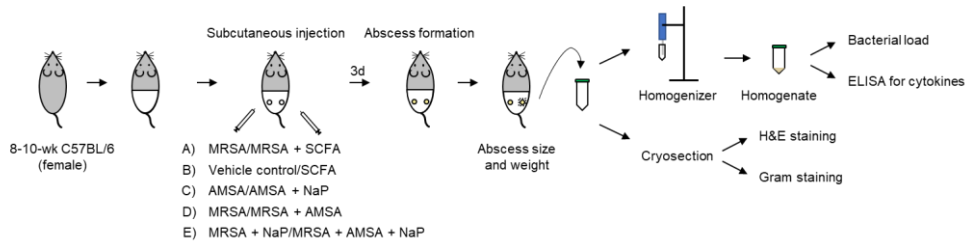

Figure 2. General experimental scheme of *in vivo* skin infection experiments. C57BL/6 mice were anesthetized and shaved in the flank area. Mice were subcutaneously infected (A) with 3 × 10^6 CFU MRSA alone, or with acetate, propionate, or butyrate, (B) with endotoxin-free water (vehicle control) or with propionate only, (C) with AMSA or AMSA and propionate, (D) with 3 × 10^6 CFU MRSA with or without AMSA alone, (E) with 3 × 10^6 CFU MRSA with propionate or with propionate and AMSA in a volume of 100 µl. Animals were monitored daily for three days. On day 3, after euthanasia, abscess length and width were measured. Abscesses were aseptically excised and weighed. Abscesses were homogenized, serially diluted, and plated on TSB agar plates to measure bacterial load. Homogenates of abscesses were centrifuged twice and the supernatants were used to measure IL-1β and IL-6 by ELISA. For histological analysis, skin abscesses were excised and cryosectioned longitudinally onto slide glasses. The sections were analyzed by hematoxylin and eosin (H&E) staining or Gram-staining. NaP, propionate.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Three days after mouse skin infection, abscesses were homogenized. The homogenates were centrifuged twice and the supernatants were stored at -80°C until use. IL-1β and IL-6 in the supernatants were measured using commercial ELISA kits (Biolegend, San Diego, CA, USA).

2.8. Histological analysis

Three days after mouse skin infection, abscesses were excised and cryosectioned longitudinally onto slide glasses at 10 μm using a cryocut microtome 1860 (Leica, Wetzlar, Germany). The sections were analyzed by H&E staining or Gram staining.

2.9. Statistical analysis

All *in vitro* experiments were conducted at least three times. For each experiment, the mean value ± standard deviation (SD) were obtained from triplicate samples. For *in vivo* studies, data are represented as mean values ± standard error of mean (SEM). *In vivo* data were analyzed with GraphPad Prism software. Statistical significance was measured using the paired student's *t*-test to compare between the groups. Asterisks indicate statistically significant differences; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Chapter III. Results

3.1. SCFAs inhibit the growth of MRSA

To determine if SCFAs have effects on the growth of *S. aureus*, MRSA was cultured in the presence of different doses of acetate, propionate, or butyrate, and the growth was examined. Acetate had a minimal impact on the growth of MRSA, while propionate and butyrate inhibited its growth in a dose-dependent manner (Fig. 3A-C). Interestingly, the inhibitory property of propionate was maintained until 12 h after treatment, whereas the inhibitory effect of butyrate was not (Fig. 3B, C). Butyrate only prolonged the lag phase. Next, to investigate if the effects of SCFAs are bacteriostatic or bactericidal, the MIC/MBC test was conducted with the microdilution method. The effect of acetate was minimal and there was no MIC in the concentration range tested (Fig. 3D). The MICs of propionate and butyrate were 250 and 500 mM, respectively (Fig. 3E, F). There was no MBC for all three SCFAs, indicating that SCFAs do not kill MRSA. To confirm that propionate, which was the most potent, also had a bacteriostatic effect, the MIC/MBC test was conducted with higher concentrations of propionate. There was no MBC even at 3 M propionate (Fig. 4A). Since agents are considered bacteriostatic when the MBC is greater than four times the MIC [57], propionate had a bacteriostatic effect. Moreover, when the morphology of *S. aureus* in the presence or absence of propionate was analyzed with scanning electron microscopy, *S. aureus* treated with propionate did not have morphological differences compared to non-treated (NT) *S. aureus* (Fig. 4B). In addition, propionate did not affect the pH of the extracellular medium, and the pH was stably maintained above 6 throughout the experiment (Fig. 5). To extend our

observations, different strains of clinically isolated antibiotic-resistant *S. aureus* strains were cultured in the presence or absence of propionate or butyrate. Propionate and butyrate inhibited the growth of *S. aureus*, including MRSA and VISA isolated from various sites (Table 2). Similar to the results of Fig. 3, butyrate prolonged the lag phase of MRSA. The inhibitory effect of propionate was maintained until stationary phase for most strains, while that of butyrate was not. These results suggest that propionate most potently inhibits the growth of MRSA.

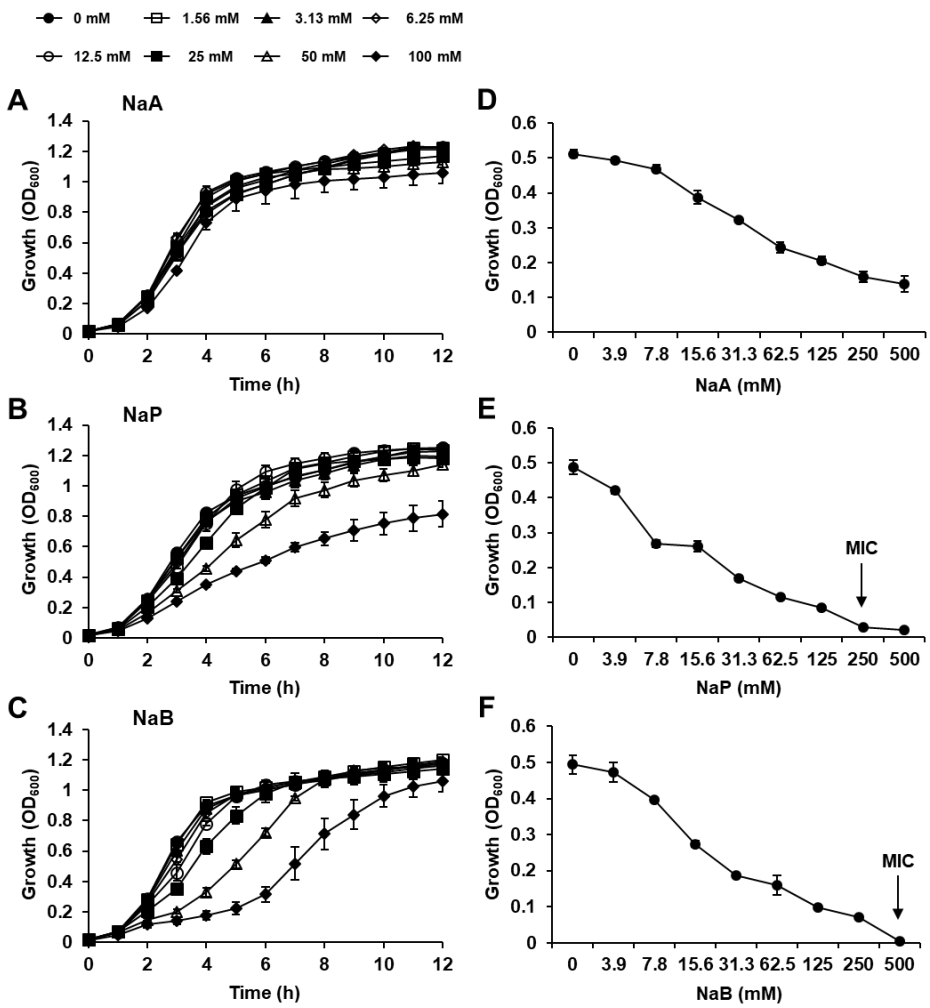

Figure 3. SCFAs dose-dependently attenuate the growth of MRSA. MRSA was cultured in the presence or absence of different doses of (A) acetate, (B) propionate, or (C) butyrate. Optical density at 600 nm was measured. (D-E) The MIC/MBC test was conducted using the microdilution method with (D) acetate, (E) propionate, or (F) butyrate. The MIC, the concentration of propionate or butyrate which completely inhibited growth, is indicated with an arrow. Data shown are the mean values ± SD of triplicate samples and are representative of at least three similar independent experiments. NaA, acetate; NaP, propionate; NaB, butyrate.

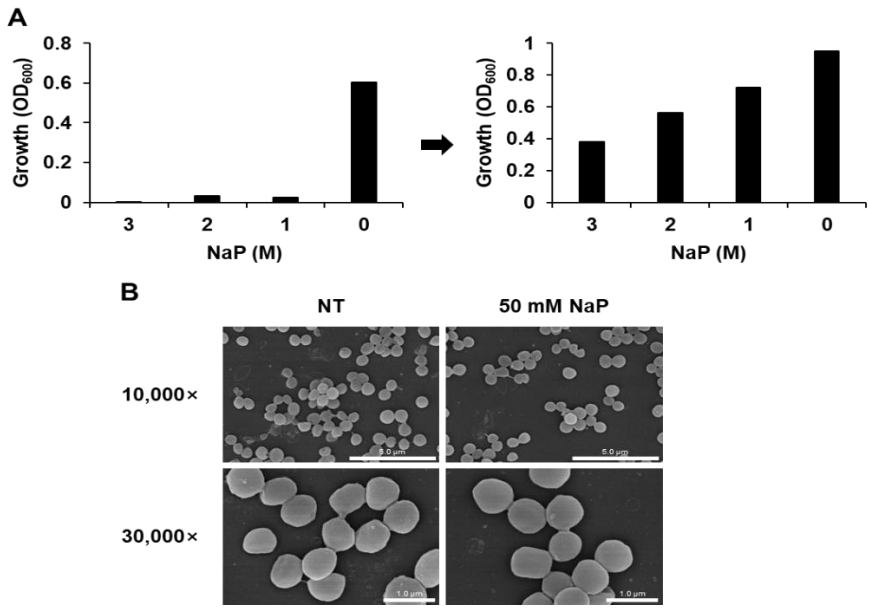

Figure 4. Propionate has a bacteriostatic effect, and does not affect bacterial morphology. (A) The MIC/MBC test was conducted with the microdilution method with 1, 2, and 3 M propionate. Optical density at 600 nm was measured after 24 h (*left*). Wells that did not show growth were inoculated in propionate-free media, and optical density was measured after an additional 24 h (*right*). The MBC was defined as the lowest concentration of propionate that resulted in no growth after inoculation in propionate-free media. Data shown are the mean values ± SD of triplicate samples and are representative of at least three similar independent experiments. (B) An overnight culture of *S. aureus* RN4220 was cultured until mid-log phase. The bacterial optical density was set to 0.5. Then, *S. aureus* was grown overnight in the absence (*left panels*) or presence (*right panels*) of 50 mM propionate. The samples were fixed, dehydrated, sputter coated with platinum and then observed with the scanning electron microscope at 10,000× (*upper panels*) and 30,000× (*lower panels*) magnification. Scale bars indicate 5 μm and 1 μm for upper and lower panels, respectively. NaP, propionate.

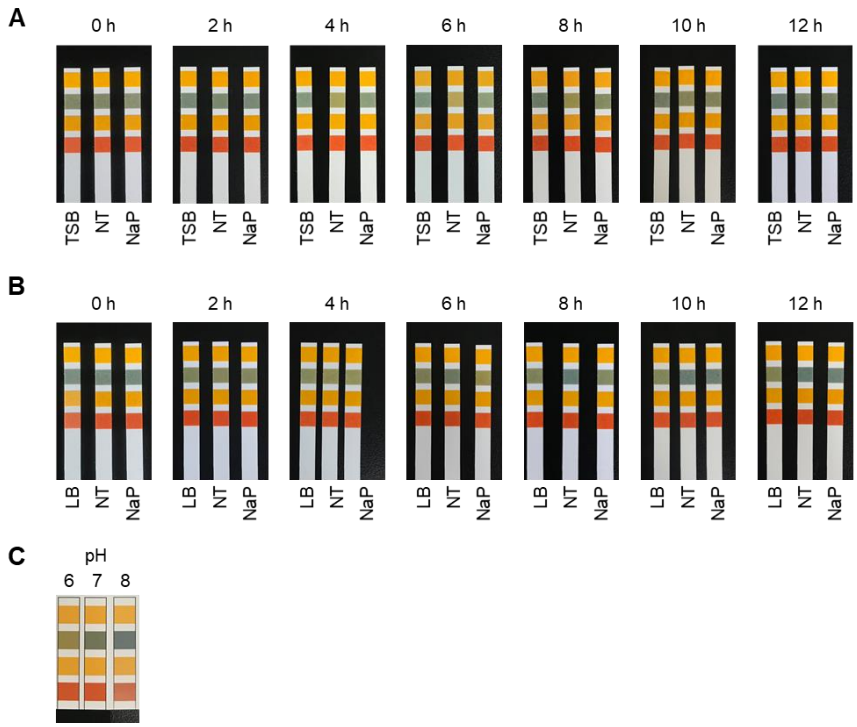

Figure 5. The pH of the extracellular medium is not greatly affected by propionate. The pH of the extracellular medium was measured (A) when MRSA USA300 was cultured in the presence of absence of 50 mM propionate or (B) when *S. aureus* RN4220 was cultured in the presence or absence of 12.5 mM propionate, with pH indicator strips throughout the experiment. (C) The reference pH strips indicating pH 6-8. NaP, propionate.

Table 2. The effects of propionate and butyrate on the growth of clinically isolated, antibiotic-resistant *S. aureus*

Strain (NCCP)	Mid-log phase[a]			Stationary phase[a]		
	NT	50 mM NaP	50 mM NaB	NT	50 mM NaP	50 mM NaB
Methicillin-resistant *S. aureus* (MRSA)						
11485[†]	0.456	0.222	0.105	1.010	0.700	0.427
11486[†]	0.462	0.221	0.164	0.950	1.001[b]	1.022[b]
14565[†]	0.844	0.423	0.265	1.061	0.822	1.027[b]
14566[†]	0.755	0.379	0.184	0.930	0.805	0.867
14567[†]	0.444	0.192	0.111	0.860	0.485	0.693
14568[†]	0.438	0.220	0.180	0.892	0.947[b]	0.920[b]
14569[†]	0.763	0.477	0.269	0.966	0.937[b]	1.044[b]
14748[†]	0.594	0.283	0.351	1.037	0.610	1.017[b]
14750[†]	0.805	0.428	0.275	0.995	0.900	1.026[b]
14751[†]	0.447	0.245	0.194	0.901	0.874	0.896[b]
14769	0.489	0.290	0.199	0.898	0.887[b]	0.904[b]
Vancomycin intermediate-resistant *S. aureus* (VISA)						
13846[†]	0.498	0.247	0.108	1.010	0.727	0.556
13853[†]	0.684	0.375	0.645[b]	0.999	0.848	1.047[b]
13863[†]	0.674	0.384	0.343	1.089	0.691	0.949

[a]OD_{600} of the same culture; [b]not significant compared to NT ($P > 0.05$)

[†]multidrug resistant

NaP, propionate; NaB, butyrate

3.2. Propionate alleviates the pathology of MRSA in skin infection

As SSTIs represent approximately 90% of MRSA infections [3], the effects of SCFAs on MRSA infection *in vivo* were investigated by using a mouse skin infection model. Mice were subcutaneously infected with MRSA with or without acetate, propionate, or butyrate. Abscesses formed by day 3 post-infection (Fig. 6A). The abscess size and weight were significantly reduced when propionate was injected together with *S. aureus*, while acetate or butyrate did not affect abscesses under the same conditions (Fig. 6B, C). When the abscesses were excised, homogenized, serially diluted, and plated on TSB agar plates to measure bacterial load, propionate, but not acetate or butyrate, significantly lowered the bacterial load in the abscesses (Fig. 6D). Moreover, IL-1β, a signature cytokine of *S. aureus* abscess formation [5], and IL-6, a proinflammatory cytokine, were lower in abscesses co-injected with propionate than in NT abscesses (Fig. 6E, F). Interestingly, butyrate did not affect abscess size, bacterial load, or cytokine expression (Fig. 6A-F), although it had some inhibitory effects *in vitro*. Histological analysis of abscesses demonstrated high immune cell infiltration, and bacterial clusters, both indicated with an arrow, in mice infected with MRSA (Fig. 6G, *left*). In propionate-treated mice, there was less cell infiltration and a lower number of bacterial clusters (Fig. 6G, *right*). SCFAs was not toxic at the concentrations used, and did not result in pathology (Fig. 7A-D). Propionate did not induce cytokine expression in mice (Fig. 7E, F). In addition, propionate did not change the size and weight of abscesses formed by injection of heat-killed MRSA at two different doses (Fig. 8A-F). To extend our results to more reflect actual infection conditions, the effects of propionate when treated after MRSA infection was initiated were investigated. When propionate was administered at 1,

24, and 48 h.p.i. via an intra-abscess injection, propionate treatment reduced abscess size and weight (Fig. 9A-C). Although the bacterial load slightly decreased when propionate was treated, it did not decrease statistically significantly ($P = 0.1547$) (Fig. 9D). These results suggest that propionate ameliorates MRSA skin infection, and reduces abscess formation even when treated after the infection is initiated.

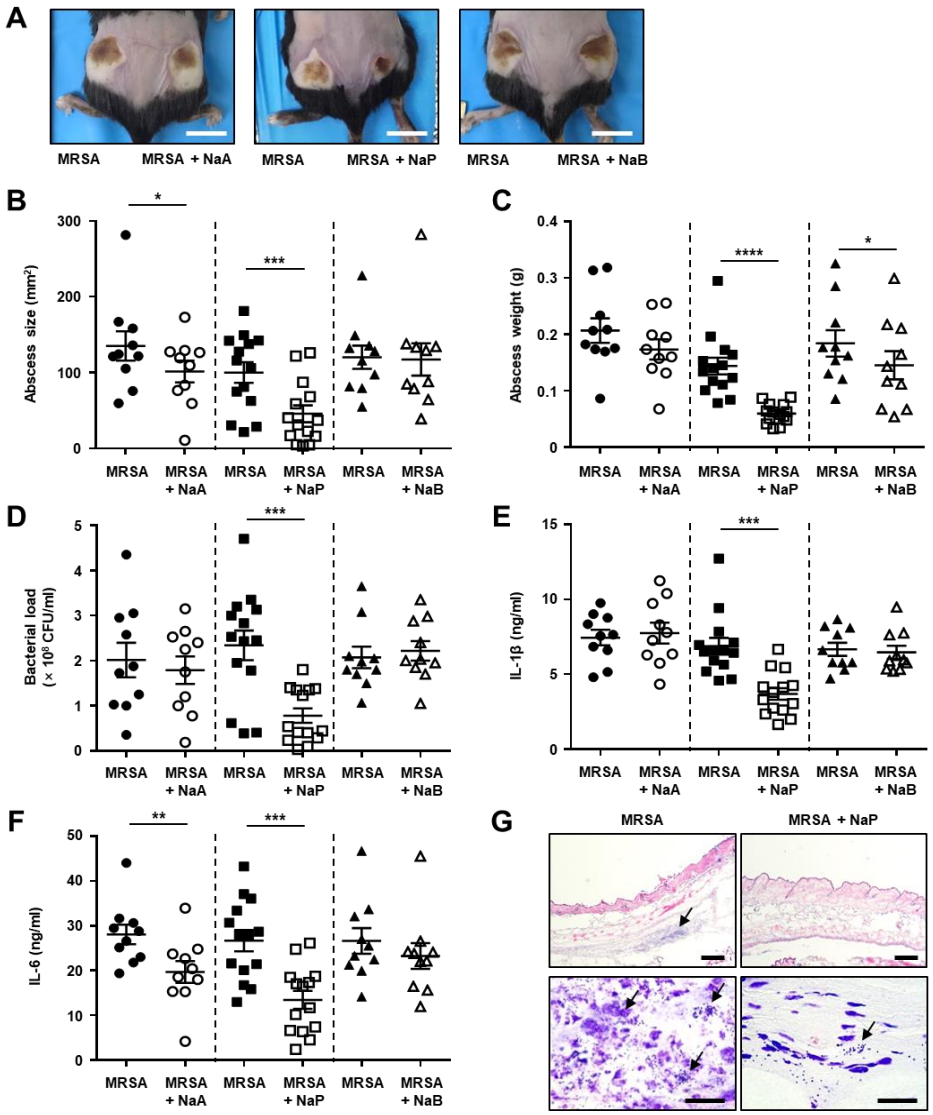

Figure 6. Propionate reduces bacterial load and dermonecrosis in mouse MRSA skin infection. C57BL/6 mice were subcutaneously infected with 3 × 10^6 CFU MRSA USA300 alone (*left*), or together with 50 mM acetate, propionate, or butyrate (*right*) (n = 10-14 per group). (A) Images of abscesses on day 3. Scale bars indicate 1 cm. On day 3, after euthanasia of mice, (B) size and (C) weight of abscesses were measured. (D) Bacterial load was measured by excising and homogenizing abscesses aseptically, and spotting homogenates on TSB agar plates. Homogenates were

centrifuged and the supernatants were used to measure (E) IL-1β and (F) IL-6. (G) Abscesses were cryosectioned and evaluated for histopathology by H&E staining and Gram staining. Scale bars indicate 200 μm and 20 μm for top and bottom panels, respectively. Data are represented as mean values ± SEM, and statistical significance was measured with the paired student's *t*-test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$. NaA, acetate; NaP, propionate; NaB, butyrate.

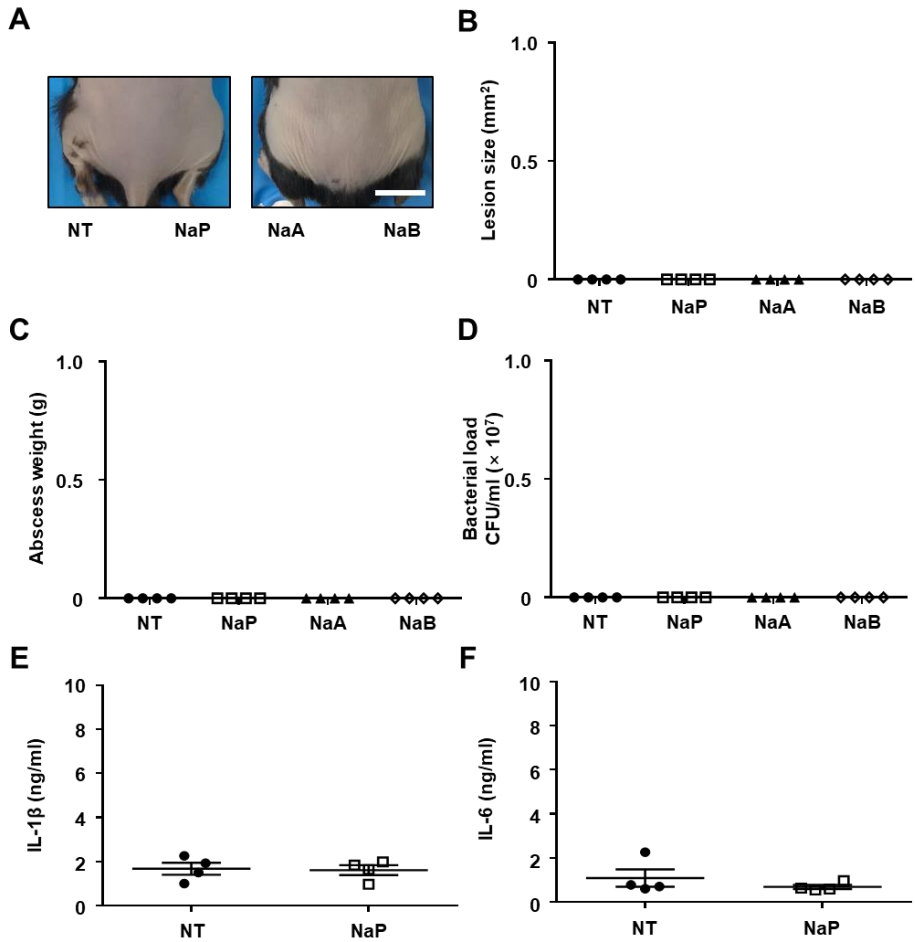

Figure 7. SCFAs do not cause pathology *in vivo*. C57BL/6 mice were subcutaneously injected with endotoxin-free water, or 50 mM propionate, acetate, or butyrate (n = 4). (A) Images of injected area on day 3. Scale bar indicates 1 cm. There was no abscess formation so (B) abscess size and (C) abscess weight could not be measured. (D) The injected areas were excised, homogenized, and plated on TSB agar plates to measure bacterial load. Homogenates for endotoxin-free water or propionate were centrifuged and supernatants were used to measure (E) IL-1β and (F) IL-6 for endotoxin-free water and propionate. Data are represented as mean values ± SEM, and statistical significance was measured with the paired student's *t*-test. NaP, propionate; NaA, acetate; NaB, butyrate.

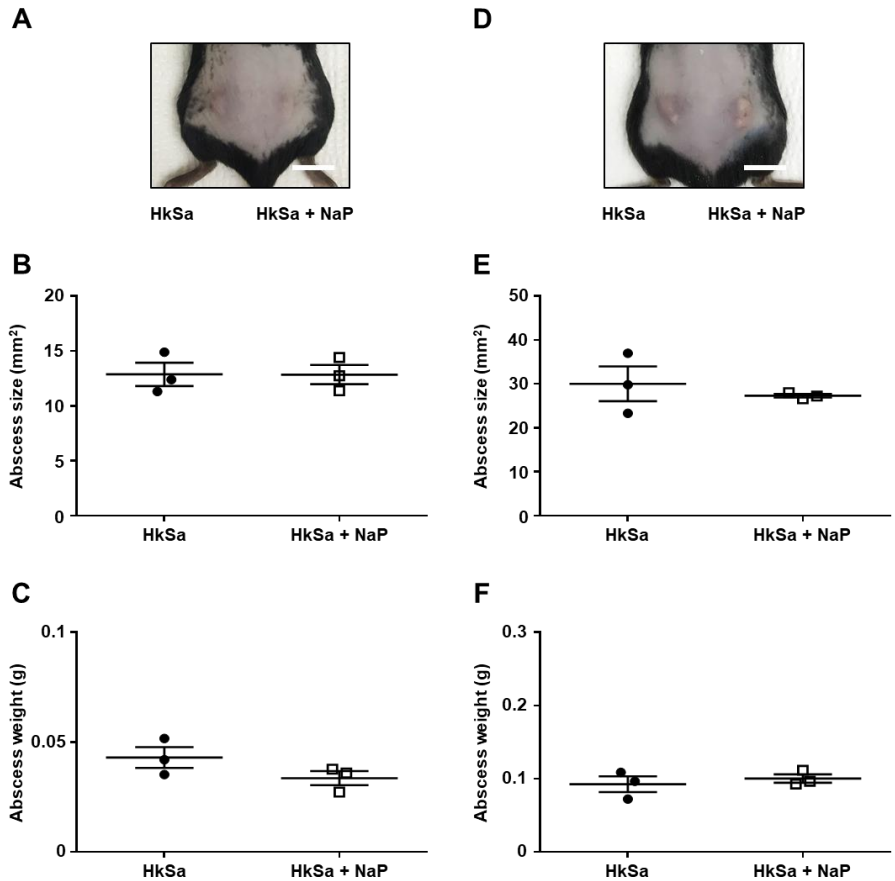

Figure 8. Propionate does not affect abscess formation by heat-killed MRSA. C57BL/6 mice (n = 3) were subcutaneously injected with (A-C) 1×10^8, or (D-F) 1×10^9 CFU heat-killed MRSA alone, or with 50 mM propionate. (A, D) Images of abscesses on day 3. (B, E) Abscess size and (C, F) abscess weight were measured. Data are represented as mean values ± SEM, and statistical significance was measured with the paired student's t-test. HkSa, heat-killed MRSA. NaP, propionate.

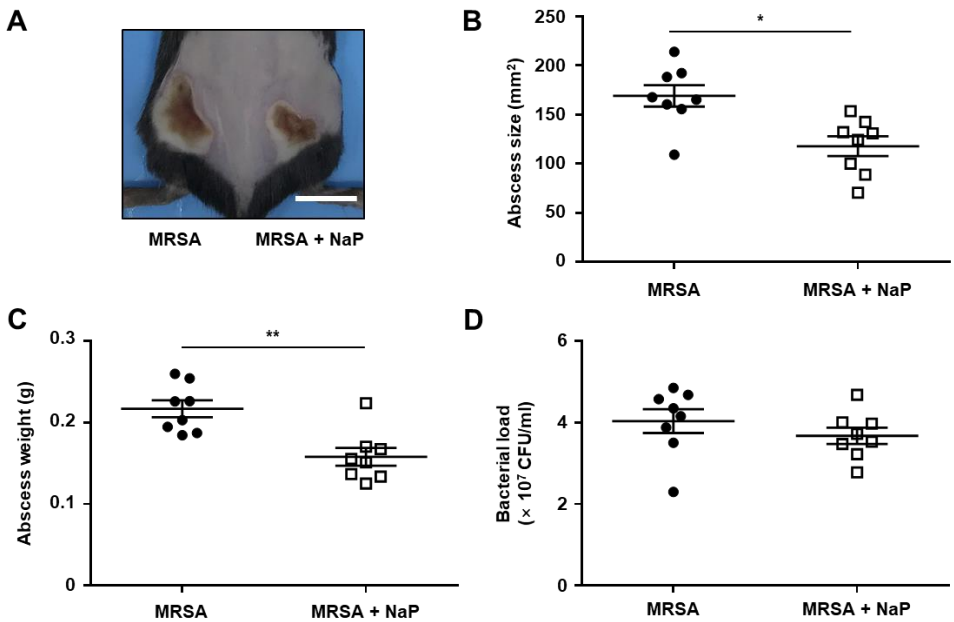

Figure 9. Propionate reduces abscess formation when treated after MRSA infection is initiated. (A) C57BL/6 mice (n = 8) were subcutaneously infected with 3 × 10^6 CFU MRSA USA300. Propionate (50 mM) was injected directly into the abscess at 1, 24, and 48 h post-infection (h.p.i.). Endotoxin-free distilled water was administered as vehicle control. On day 3, (B) abscess size and (C) abscess weight were measured. (D) Abscesses were excised, homogenized, and plated on TSB agar plates to measure bacterial load. Data are represented as mean values ± SEM, and statistical significance was measured with the paired student's t-test. *, $P < 0.05$, **, $P < 0.01$. NaP, propionate.

3.3. *S. aureus* with $_{D}$-alanine-deficient LTA and WTA is more susceptible to the growth inhibition by propionate

In Gram-positive bacteria, cell wall components such as LTA, WTA and LPP play important roles in bacterial growth, division, and antimicrobial susceptibility (Fig. 10) [58-60]. To gain insight into the action mechanism of the growth inhibition by propionate, the effects of SCFAs on the growth of WT, Δ*ltaS*, Δ*tagO*, Δ*lgt S. aureus* (Fig. 10C) were compared. First, the dose-dependent effects of acetate, propionate, or butyrate on WT *S. aureus* were confirmed (Fig. 11A-C). Compared to the WT, LTA-deficient or WTA-deficient *S. aureus* was more susceptible to the growth inhibition by propionate (Fig. 12A-C), while LPP-deficient *S. aureus* exhibited a similar growth pattern (Fig. 12D). Complement strains of each mutant had more similar growth patterns compared to the WT (Fig. 12E-G). Since LTA and WTA share $_{D}$-alanine motifs in common [61], the effects of SCFAs on the growth of *S. aureus* which lacks $_{D}$-alanine motifs on teichoic acids, which are important for antimicrobial peptide and antibiotic resistance [62, 63], were examined. Interestingly, $_{D}$-alanylation-deficient *S. aureus* was substantially more susceptible to propionate than the WT, its growth being almost completely inhibited (Fig. 13A, B). Moreover, the MIC of propionate for $_{D}$-alanylation-deficient *S. aureus* was 62.5 mM, a value four-fold lower than that for the WT (Fig. 13D, E). As expected, the complement strain had similar growth patterns as the WT (Fig. 13C) and had an equal MIC value (Fig. 13F). Therefore, $_{D}$-alanine motifs of teichoic acids are important in modulating the susceptibility of *S. aureus* to propionate.

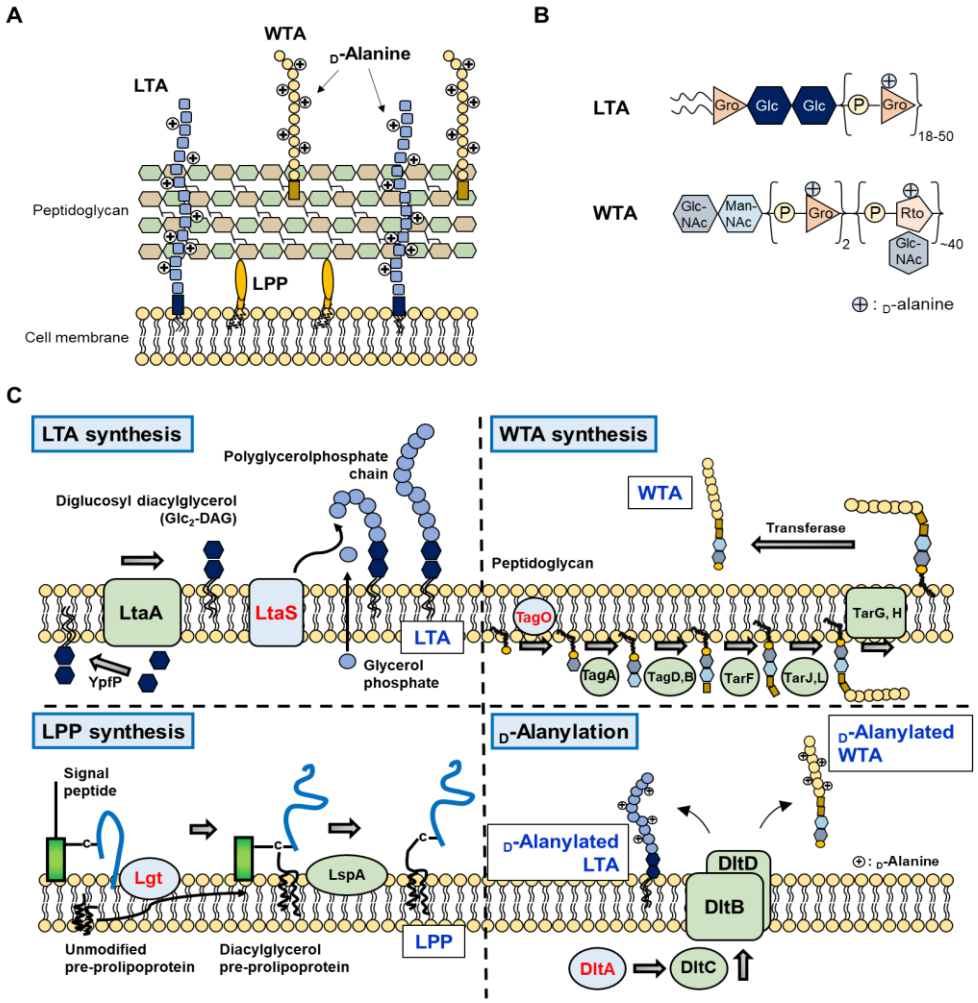

Figure 10. Cell wall components of *S. aureus*. (A) The major cell wall components of *S. aureus* are LTA, WTA, and LPP. LTA, which is linked to the cell membrane, plays an important role in cell division and membrane homeostasis. WTA is covalently linked to the peptidoglycan, and plays an important role in cell division and antibiotic resistance. LPP is anchored to the membrane and is important for physiology and nutrient acquisition. D-Alanine motifs, on both LTA and WTA, play a role in antimicrobial peptide resistance. (B) Schematic structures of LTA and WTA. Gro, glycerol; Glc, glucose; P, phosphate; +, D-alanine; GlcNAc, N-acetylglucosamine; ManNAc, N-acetylmannosamine, Rto, ribitol. (C) Simplified

schematic of the synthesis steps of each cell wall component. LTA is synthesized by LtaA and LtaS, which form the polyglycerolphosphate chain. WTA is synthesized by Tag and Tar enzymes, which connect glycerol or ribitol groups. LPP is synthesized via Lgt and Lsp enzymes. The *dltABCD* operon is responsible for $_{D}$-alanylation of both LTA and WTA. Mutant strains deficient of an enzyme in each process (in red) were used.

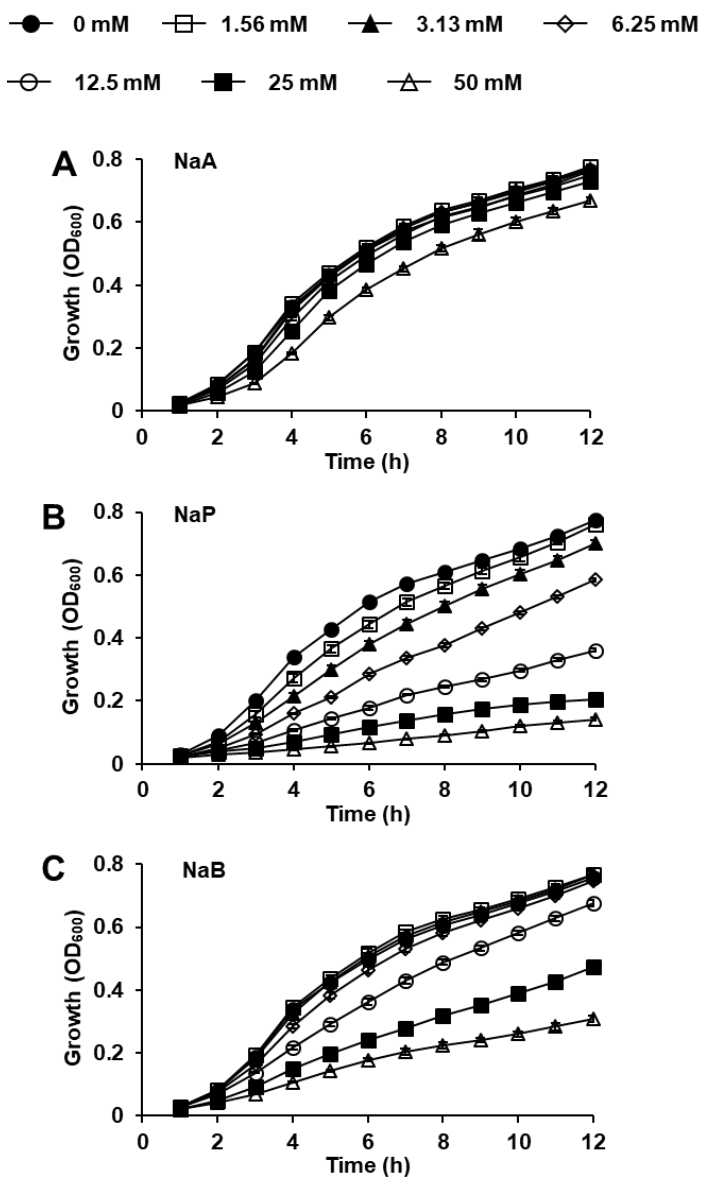

Figure 11. Propionate potently inhibits the growth of *S. aureus* in a dose-dependent manner. *S. aureus* RN4220 was cultured with different doses of (A) acetate, (B) propionate, or (C) butyrate. The optical density at 600 nm was measured. Data shown are the mean values ± SD of triplicate samples and are representative of at least three similar independent experiments. NaA, acetate; NaP, propionate; NaB, butyrate.

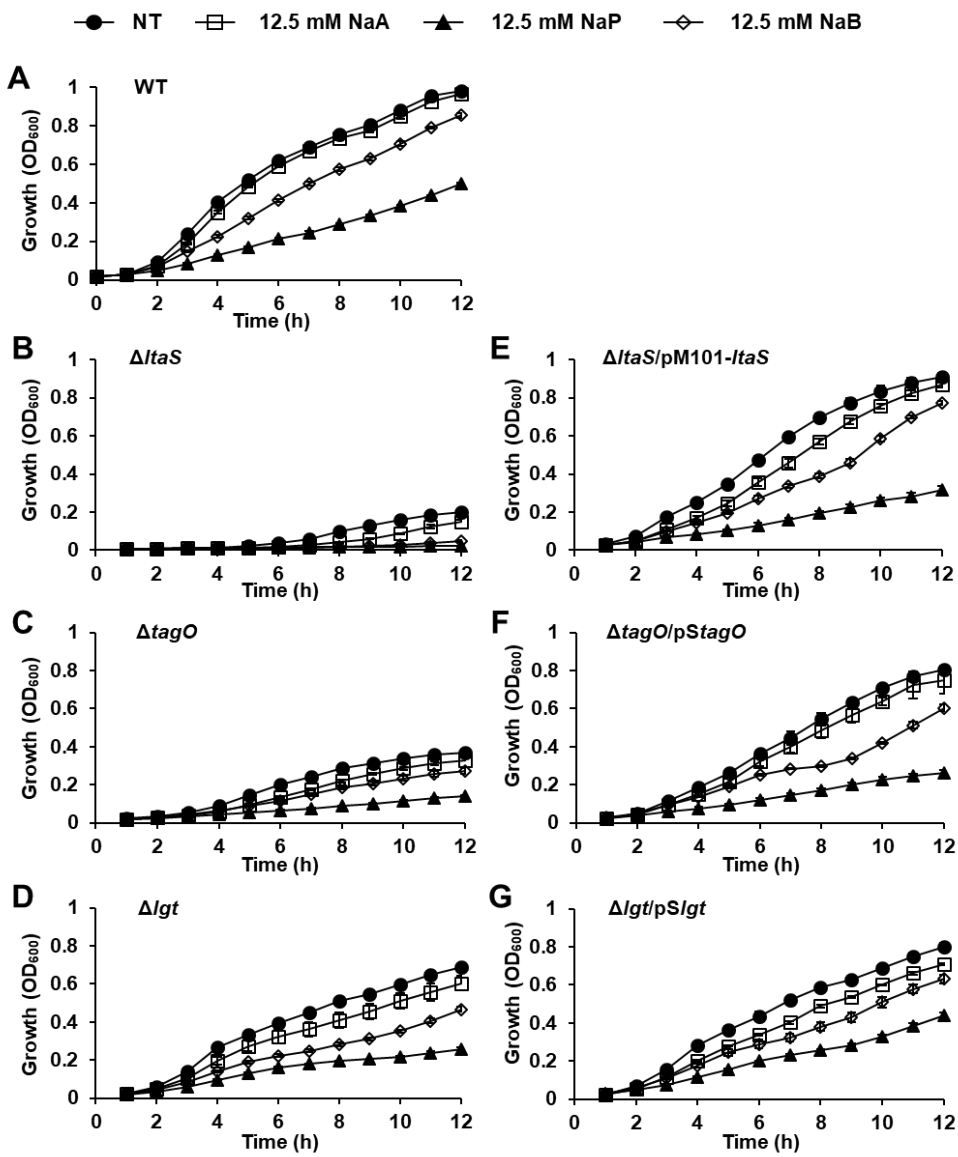

Figure 12. LTA-deficient or WTA-deficient *S. aureus* is susceptible to the growth inhibition by propionate. (A) WT (B) Δ*ltaS*, (C) Δ*tagO*, (D) Δ*lgt*, (E) Δ*ltaS*/pM101-*ltaS*, (F) Δ*tagO*/pS*tagO*, or (G) Δ*lgt*/pS*lgt* was inoculated and cultured in the presence or absence of 12.5 mM of acetate, propionate, or butyrate. The optical density at 600 nm was measured. Data shown are the mean values ± SD of triplicate samples and are representative of at least three similar independent experiments. NaA, acetate; NaP, propionate; NaB, butyrate.

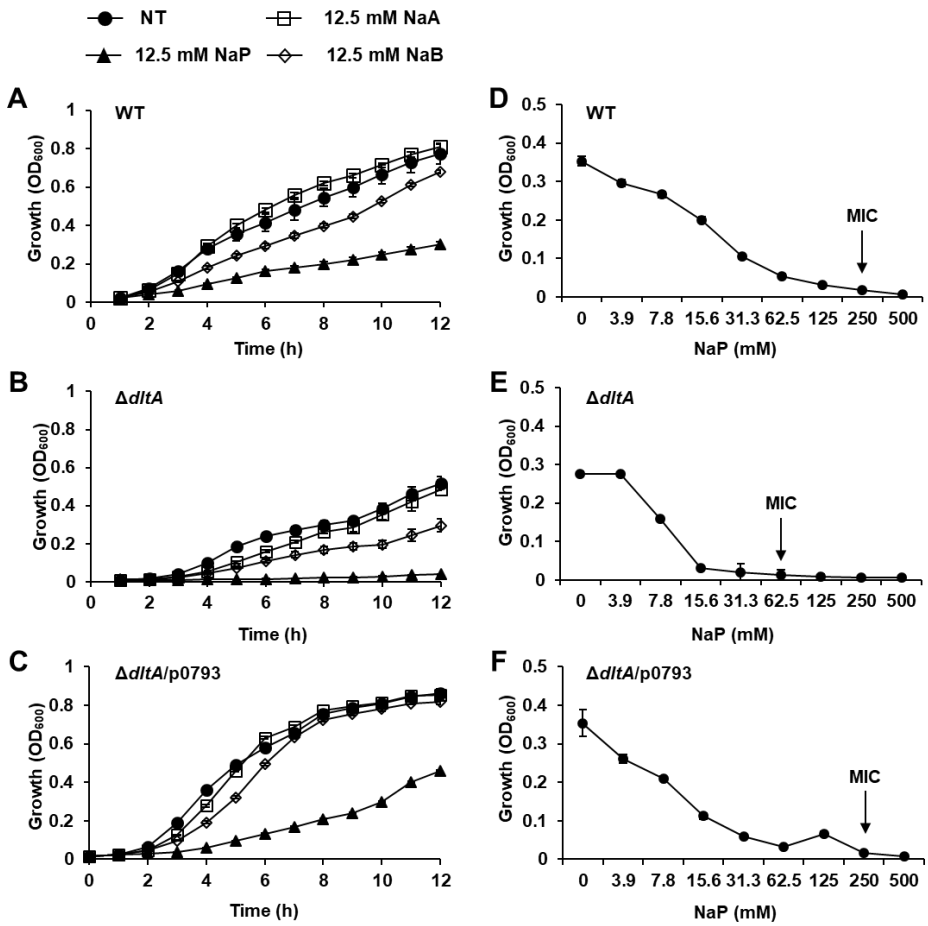

Figure 13. *S. aureus* with D-alanine-deficient LTA and WTA is susceptible to the growth inhibition by propionate. (A) WT (B) Δ*dltA*, or (C) Δ*dltA*/p0793 was inoculated and cultured in the presence or absence of 12.5 mM of acetate, propionate, or butyrate. The optical density at 600 nm was measured. (D-F) The MIC/MBC test for propionate was conducted using the microdilution method with (D) WT, (E) Δ*dltA*, or (F) Δ*dltA*/p0793. The MIC, the concentration of propionate which completely inhibited growth, is indicated with an arrow. Data shown are the mean values ± SD of triplicate samples and are representative of at least three similar independent experiments. NaA, acetate; NaP, propionate; NaB, butyrate.

3.4. $_{D}$-Alanylation inhibition increases susceptibility of MRSA to the growth inhibition by propionate

Next, the importance of $_{D}$-alanine residues in the growth inhibition by propionate was also investigated in MRSA. A $_{D}$-alanylation inhibitor, AMSA, which inhibits DltB, the transmembrane involved in $_{D}$-alanylation of teichoic acids [64], was used. When MRSA was cultured in the presence of different concentrations of AMSA, the growth of MRSA was minimally affected upto 25 μg/ml (Fig. 14A). The MIC of AMSA was 100 μg/ml (Fig. 14B). AMSA was used at 10 μg/ml, a concentration that inhibits $_{D}$-alanylation, but has minimal effects on bacterial growth, for combination experiments. Co-treatment of 10 μg/ml AMSA and 50 mM propionate resulted in approximately 80% inhibition of MRSA growth (Fig. 14C). Moreover, the MIC of propionate was reduced two-fold to 125 mM when AMSA was co-treated (Fig. 14D). In addition, analysis with the checkerboard assay for the FICI demonstrated that propionate and AMSA have partial synergy (FICI = 0.6). These results indicate that $_{D}$-alanine residues on teichoic acids are important in modulating the susceptibility of MRSA to propionate.

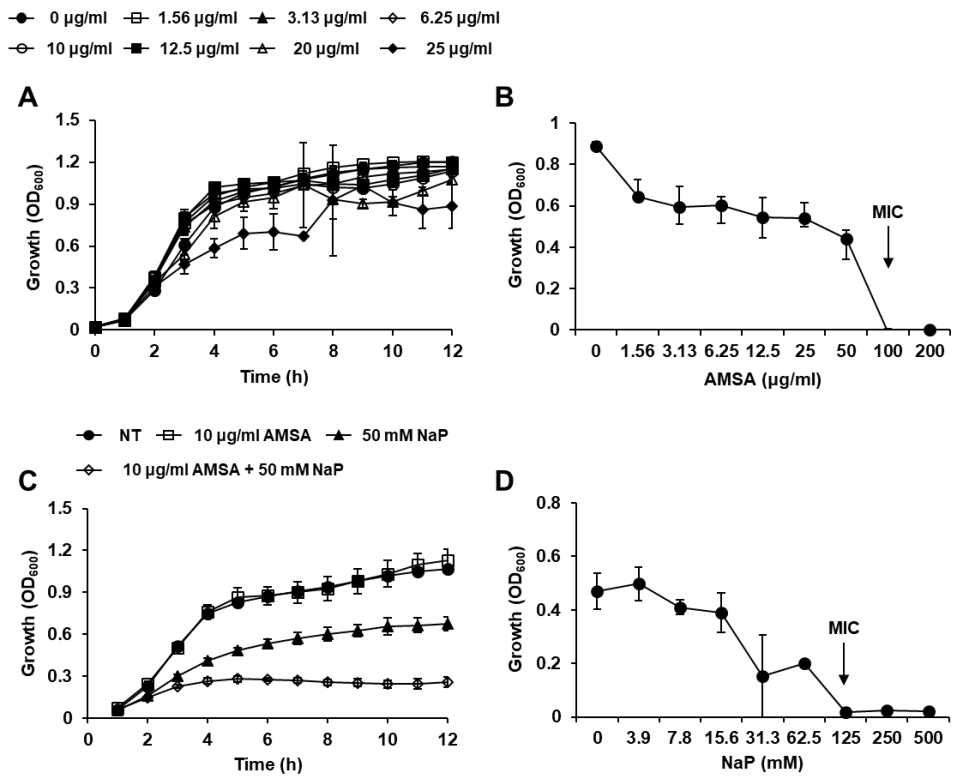

Figure 14. $_{D}$-Alanylation inhibition increases the susceptibility of MRSA to the growth inhibition by propionate. (A) MRSA was cultured in the presence of different doses of AMSA, a $_{D}$-alanylation inhibitor. Optical density at 600 nm was measured. (B) The MIC/MBC test was conducted using the microdilution method with AMSA. The MIC, the concentration of propionate which completely inhibited growth, is indicated with an arrow. (C) MRSA USA300 was cultured in the presence of 10 μg/ml AMSA, 50 mM propionate, or both. Optical density at 600 nm was measured. (D) The MIC/MBC test was conducted using the microdilution method with propionate in the presence of 10 μg/ml AMSA. The MIC, the concentration of propionate which completely inhibited growth, is indicated with an arrow. Data shown are the mean values ± SD of triplicate samples and are representative of at least three independent experiments. NaP, propionate.

3.5. Co-treatment of a D-alanylation inhibitor and propionate further ameliorates MRSA skin infection

Since the co-treatment of AMSA and propionate led to almost complete inhibition of MRSA *in vitro*, their effects were also studied *in vivo*. To confirm that the concentration of AMSA used in this study is not toxic, mice were injected with AMSA only, or with AMSA and propionate. AMSA at 10 μg/ml was not toxic *in vivo* and did not result in pathology or cytokine expression (Fig. 15). In addition, as control, the effects of AMSA alone was studied by comparing mice infected with MRSA and AMSA to those infected with MRSA alone. Moreover, although AMSA slightly decreased the abscess size, it did not affect abscess weight, bacterial load, and cytokine expression (Fig. 15). To determine the effect of combination treatment, mice were subcutaneously infected with MRSA together with propionate, or with AMSA and propionate. Co-injection of AMSA and propionate further reduced abscess size and weight compared to injection of propionate alone (Fig. 16A-C). Furthermore, combination of AMSA and propionate further reduced the bacterial load and excessive cytokine expression (Fig. 16D-F). Therefore, these results indicate that co-treatment of AMSA and propionate further ameliorates MRSA skin infection, and suggest combination treatment as an efficient strategy.

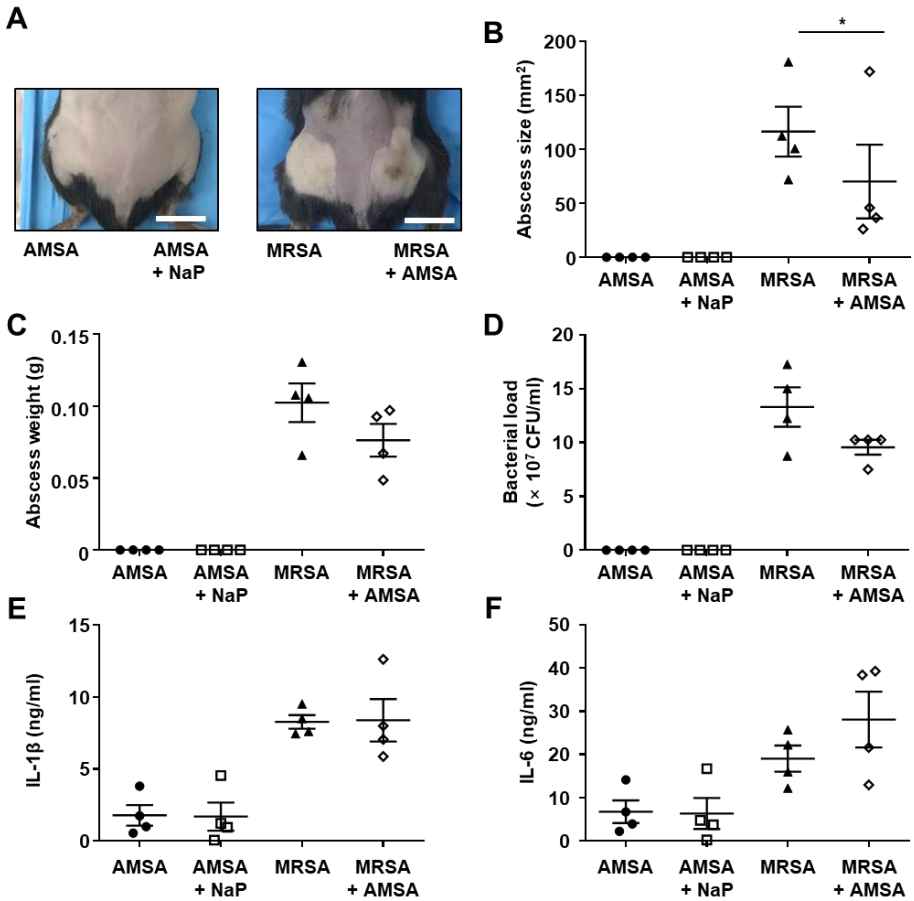

Figure 15. AMSA is not toxic *in vivo*, and AMSA alone does not reduce the pathology of MRSA infection. C57BL/6 mice were subcutaneously injected with AMSA, AMSA + propionate, MRSA, or MRSA + AMSA (n = 4). (A) Images of injection sites on day 3. Scale bar indicates 1 cm. Then, after euthanasia of mice, (B) the size and (C) weight of abscesses were measured. (D) Bacterial load was measured by excising and homogenizing abscesses aseptically, and spotting homogenates on TSB agar plates. Homogenates were centrifuged and the supernatants were used to measure (E) IL-1β and (F) IL-6 by ELISA. Data are represented as mean values ± SEM, and statistical significance was measured with the paired student's t test. *, $P < 0.05$. NaP, propionate.

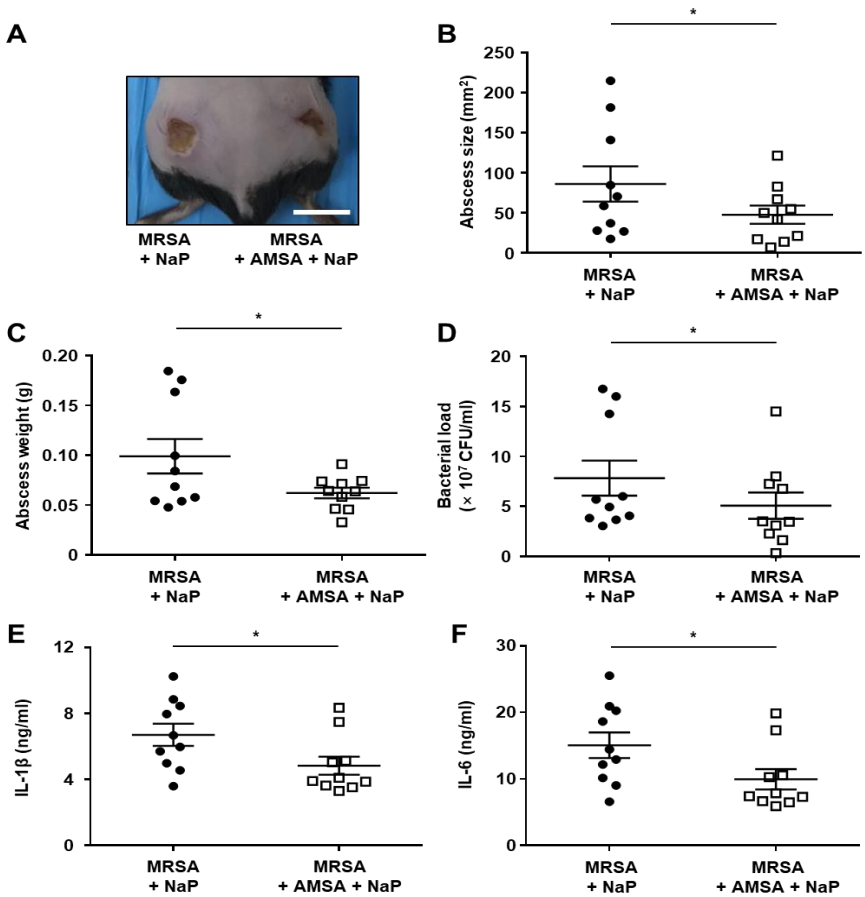

Figure 16. Propionate reduces bacterial load in murine skin infection when co-treated with a D-alanylation inhibitor. C57BL/6 mice were subcutaneously infected with 3 × 10^6 CFU MRSA USA300 together with 50 mM propionate (*left*), or with 50 mM propionate and 10 μg/ml AMSA (*right*) (n = 10). (A) Images of abscesses on day 3. Scale bar indicates 1 cm. On day 3, after euthanasia, (B) size and (C) weight of abscesses were measured. (D) Bacterial load was measured by excising and homogenizing abscesses aseptically, and spotting homogenates on TSB agar plates. Homogenates were centrifuged and the supernatants were used to measure (E) IL-1β and (F) IL-6. Data are represented as mean values ± SEM, and statistical significance was measured with the paired student's t-test. *, $P < 0.05$. NaP, propionate.

3.6. Propionate interferes with *S. aureus* metabolism

Bacteriostatic agents are likely to change the metabolic state of the bacterium, attenuating cellular respiration [65]. In *S. aureus*, the central metabolic pathway involves glycolysis, gluconeogenesis, and tricarboxylic acid (TCA) cycle (Fig. 17) [66]. To investigate the mechanisms by which propionate inhibits the growth of *S. aureus*, the effects of propionate on MRSA deficient of metabolic enzymes were studied. Compared to the WT (Fig. 18A), MRSA deficient of a gluconeogenesis enzyme was more susceptible to propionate (Fig. 18B), while MRSA deficient of a glycolytic enzyme was not (Fig. 18C). Moreover, MRSA deficient of TCA cycle enzymes was more susceptible to propionate (Fig. 19), suggesting that propionate may affect bacterial metabolism.

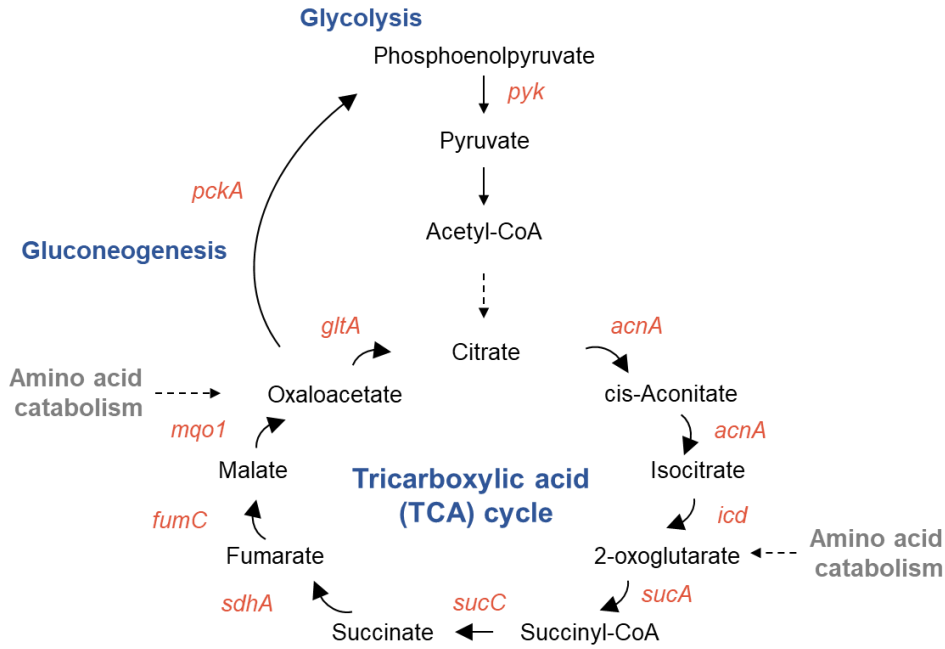

Figure 17. Central metabolic pathways of *S. aureus*. In *S. aureus*, the central carbon metabolism involves glycolysis, gluconeogenesis, and TCA cycle, along with amino acid catabolism. Genes of involved enzymes are noted.

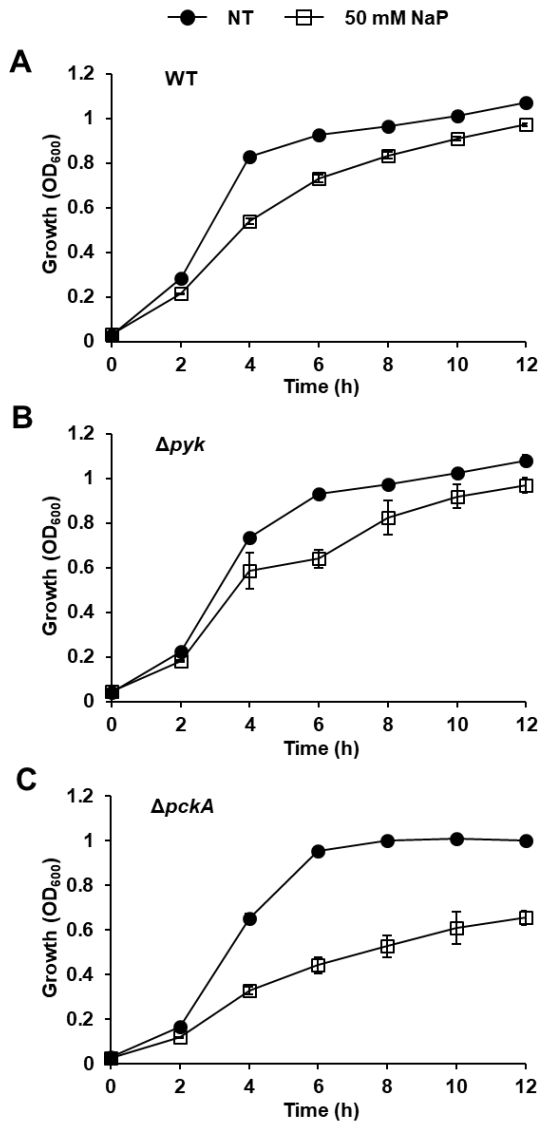

Figure 18. MRSA deficient of a gluconeogenesis enzyme is more susceptible to propionate. MRSA (A) WT, (B) glycolysis mutant (Δ*pyk*), or (C) gluconeogenesis mutant (Δ*pckA*) was cultured in the presence or absence of 50 mM propionate. Optical density at 600 nm was measured. Data shown are the mean values ± SD of triplicate samples and are representative of at least three similar independent experiments. NaP, propionate.

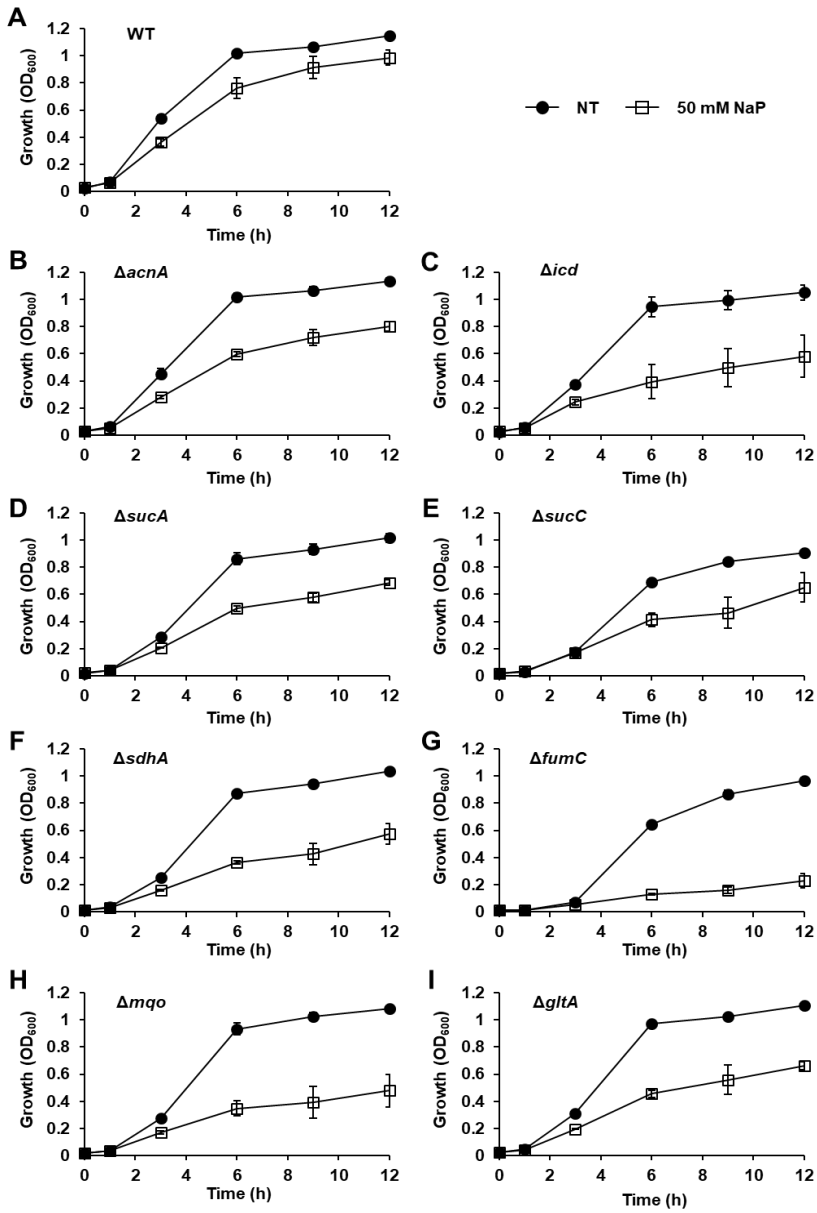

Figure 19. MRSA deficient of TCA cycle enzymes is more susceptible to propionate. MRSA (A) WT or TCA cycle mutant (B) Δ*acnA*, (C) Δ*icd*, (D) Δ*sucA*, (E) Δ*sucC*, (F) Δ*sdhA*, (G) Δ*fumC*, (H) Δ*mqo*, or (I) Δ*gltA*, was cultured in the presence or absence of 50 mM propionate. Optical density at 600 nm was measured. Data shown are the mean values ± SD of triplicate samples and are representative of at least three similar independent experiments. NaP, propionate.

3.7. Propionate inhibits the growth of other pathogens

To determine if the bacteriostatic effect of propionate is unique to *S. aureus*, the effect of propionate on other bacteria was studied. The degree of susceptibility varied among the tested bacteria. The growth of *S. pneumoniae* (Fig. 20A) or *E. faecium* (Fig. 20B) was potently inhibited, while the growth of *S. epidermidis* (Fig. 20C) or *E. faecalis* (Fig. 20D) was moderately inhibited. The growth of *S. gordonii* (Fig. 20E) or *L. plantarum* (Fig. 20F) was not inhibited by propionate. Thus, the susceptibility of propionate seems to vary depending on the bacterial species. Collectively, the results of this study indicate that propionate inhibits the growth of *S. aureus* both *in vitro* and *in vivo*, and that D-alanine motifs modulate the susceptibility of *S. aureus* to propionate (Fig. 21). These suggest propionate, together with a D-alanylation inhibitor, as an alternative therapeutic strategy to control antibiotic-resistant *S. aureus* infections.

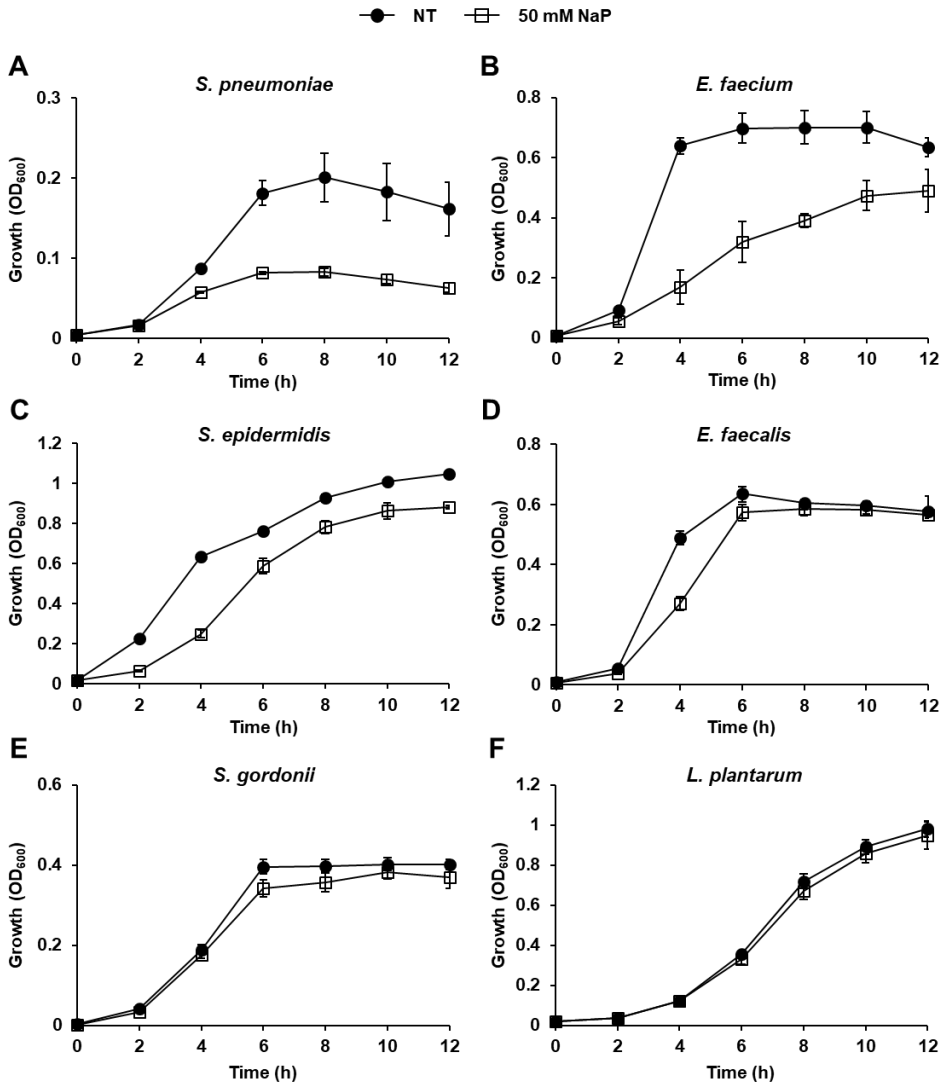

Figure 20. Propionate inhibits the growth of other pathogens. (A) *S. pneumoniae*, (B) *E. faecium*, (C) *S. epidermidis*, (D) *E. faecalis*, (E) *S. gordonii*, and (F) *L. plantarum* were cultured in the presence or absence or 50 mM propionate. Optical density at 600 nm was measured. Data shown are the mean values ± SD of triplicate samples and are representative of at least three similar independent experiments. NaP, propionate.

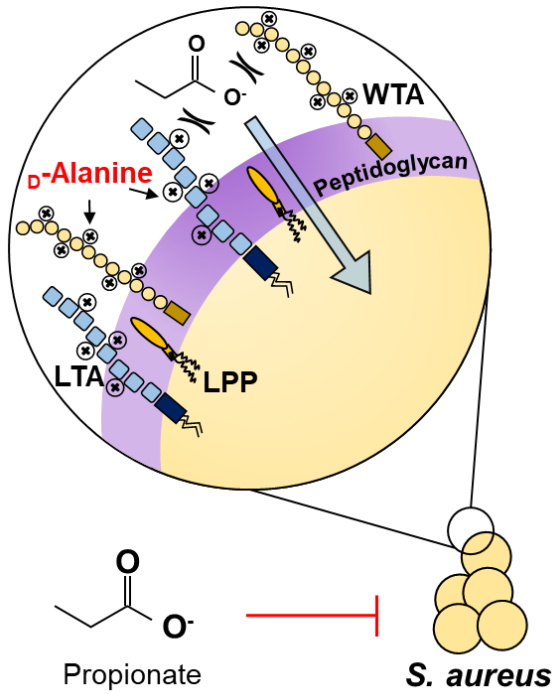

Figure 21. Schematic illustration of the proposed action mechanism of propionate. Propionate inhibits the growth of *S. aureus*. D-Alanylation-deficient *S. aureus* is more susceptible to the growth inhibition by propionate. D-Alanine motifs on teichoic acids of *S. aureus* modulate the susceptibility of *S. aureus* to propionate. Propionate is likely to be able to diffuse into *S. aureus* more readily because of the decreased cell wall density due to the absence of D-alanine motifs.

Chapter IV. Discussion

Since the prevalence in infections caused by multidrug-resistant *S. aureus* is increasing, and there is a limited availability of antibiotics along with possible side effects, alternative therapeutic strategies against *S. aureus* infections are urgently needed. To overcome multidrug-resistant *S. aureus* infections, combination therapy and the concept of synthetic lethality have been gaining more attention [25, 26]. Combination therapy has been thought to be more effective and less prone to resistance [67]. If there were synergy between the therapeutic agents, the amounts of the agents used may also be reduced. In the present study, propionate inhibited the growth of *S. aureus* including multidrug-resistant strains, likely by interfering with bacterial metabolism, and ameliorated MRSA skin infection, which represents a majority of MRSA infections. In addition, a combination treatment of propionate and AMSA, a D-alanylation inhibitor, more potently inhibited MRSA infection, suggesting combination treatment as an efficient alternative strategy for control of multidrug-resistant *S. aureus* infections.

Of the three SCFAs, propionate most potently inhibited the growth of MRSA both *in vitro* and *in vivo*. The inhibitory effect of propionate seems to be a general phenomenon in *S. aureus*, since propionate inhibited the growth of all strains of *S. aureus* tested, including clinically isolated multidrug-resistant *S. aureus*. Similarly, it has been previously suggested that the fermentation products of *P. acnes*, which contain butyric acid, 3-hydroxy-butyric acid, lactic acid, propionic acid, and ethanol, can interfere with *S. aureus* colonization in a wound model [45]. Although the

involvement of other proteins or metabolites cannot be excluded, propionic acid seems to have been responsible for colonization interference as propionic acid of pH 3.5 inhibited *S. aureus* colonization as well. In addition, another study has reported the growth inhibitory properties of propionic acid, which changes the pH of the extracellular medium [46]. Since a high concentration of propionic acid may result in an acidic environment, propionic acid could lead to side effects such as skin irritation or corrosion [68]. Therefore, in this study, propionate, which is not acidic and does not change the extracellular pH, was used. Propionate inhibited *S. aureus* growth and ameliorated MRSA skin infection without affecting the pH of the extracellular environment.

It has been suggested that bacteriostatic agents can change the metabolic state of the bacterium and interfere with its metabolism [65]. Propionate might interfere with metabolic pathways that are important for *S. aureus* growth, such as cellular respiratory pathways including glycolysis, TCA cycle, or oxidative phosphorylation. Indeed, MRSA deficient of a gluconeogenesis enzyme or a TCA cycle enzyme was more susceptible to propionate, suggesting a possible relationship. In addition, it has been reported that propionate inhibits the growth of *Rhodopseudomonas sphaeroides* by interfering with pyruvate decarboxylation [69]. The authors suggested that propionate gets converted to propionyl-CoA, which then interferes with the pyruvate dehydrogenase complex. Since *S. aureus* also has a putative propionate CoA-transferase [70], propionate may also be converted to propionyl-CoA and interfere with pyruvate decarboxylation in *S. aureus* as well. Moreover, since propionate potently inhibited the growth of *S. aureus* while acetate and butyrate did not, it is

likely that propionate would uniquely target a specific pathway that the other SCFAs do not. Acetate or butyrate may not interfere with metabolism, since acetate is produced by *S. aureus* and can enter the TCA cycle, and since butyrate can be produced through fermentative metabolism [71, 72]. Although further studies are needed to address the possible mechanisms, propionate seems to affect the metabolic pathways of *S. aureus*.

S. aureus was more susceptible to the growth inhibition by propionate when $_{D}$-alanine motifs on LTA and WTA are absent, demonstrated by using $_{D}$-alanylation-deficient *S. aureus*, and a $_{D}$-alanylation inhibitor. $_{D}$-Alanylation residues on LTA and WTA are important for many physiological processes, including regulation of autolytic enzymes, colonization, and virulence [73-75]. Furthermore, the absence of $_{D}$-alanylation leads to increased susceptibility of bacteria to antimicrobial peptides, antibiotics, and neutrophil killing [62, 63, 74]. It has been reported that $_{D}$-alanylation of teichoic acids increases the cell wall density and rigidity, and that $_{D}$-alanine residues confer resistance to antimicrobial peptides by decreasing permeability, rather than by conferring positive charges [76]. It is likely that when $_{D}$-alanine residues on teichoic acids are absent, propionate may be able to diffuse more easily into *S. aureus* to interfere with bacterial metabolism. In addition, the varying degree of susceptibility among the different strains of *S. aureus* tested may have been due to differences in the level of $_{D}$-alanylation of teichoic acids, considering that antibiotic-resistant *S. aureus* clinical isolates have been reported to have increased $_{D}$-alanylation [77]. Even though further studies are needed to elucidate the connection between propionate susceptibility and $_{D}$-alanylation, a correlation may

exist, and combination therapy of propionate and a D-alanylation inhibitor may be an effective strategy to control multidrug-resistant *S. aureus* infections. Furthermore, inhibitors that target other cell wall components such as teichoic acids may also have potential as combination therapy agents.

Propionate significantly decreased the pathology of MRSA skin infection, and lowered abscess formation, bacterial load, and excessive cytokine expression. SCFAs are well-known to exhibit immunomodulatory effects [32]. In this study, the amelioration of MRSA skin infection is more likely to have been due to the reduced absolute number of bacteria and the bacteriostatic activity of propionate, since propionate did not change the size and weight of abscess formed by subcutaneous injection of heat-killed MRSA. In addition, another bacteriostatic agent had antibacterial effects in *S. aureus* lung infection [78]. Although the immunomodulatory effect of propionate cannot be excluded, it is likely that the effect of propionate on the host immune system is more minor compared to its effect on *S. aureus* itself. Butyrate is known to more potently regulate the immune system than propionate [79, 80], but butyrate did not decrease pathology in these experimental conditions. Moreover, IL-1β and IL-6 expression decreased when the bacterial load was lower, suggesting that cytokine expression was decreased because of a reduction in the absolute number of bacteria. Although IL-1β is important for immunity against *S. aureus* infection [5], excessive IL-1β production has detrimental effects [6]. When propionate is treated, there would have been less excessive inflammation caused by IL-1β and IL-6 released from neutrophils, the key cell type in *S. aureus* skin infection. In addition, since targeting bacteria with AMSA further

ameliorated MRSA skin infection, the decreased pathology of MRSA seems to be a result of decreased bacterial load. Therefore, propionate ameliorates MRSA skin infection by attenuating bacterial growth, and the effect of propionate on the host seems to be more minor.

Interestingly, different bacterial species had varying susceptibility to propionate. For example, pathogens *S. pneumoniae* and *E. faecium* were highly susceptible, while commensals *S. epidermidis* and *E. faecalis* were moderately susceptible. Commensal *S. gordonii* and probiotic *L. plantarum* were not susceptible to 50 mM propionate. The varying effects of SCFAs depending on bacterial species have been previously suggested as well [81]. SCFAs have been suggested to inhibit the growth or virulence of pathogenic bacteria [41, 43, 81]. Coincidently, in this study, pathogenic bacteria were more susceptible to propionate than commensal or probiotic bacteria. Commensal and probiotic bacteria were less or not susceptible to the growth inhibition by propionate. Since SCFAs also exist systemically, probiotic and commensal bacteria may have adapted to be tolerant to SCFAs because they are frequently exposed to SCFAs. In addition, the varying susceptibility may be due to differences in the level of $_{D}$-alanylation of teichoic acids among the different bacterial species, considering that *S. aureus* was more susceptible to propionate when $_{D}$-alanine motifs were absent. Although this needs further investigation, propionate may selectively inhibit the growth of pathogenic bacteria.

In this study, propionate inhibited the growth of MRSA and other clinically isolated

multidrug-resistant *S. aureus*, and ameliorated MRSA skin infection. Since propionate was not toxic *in vivo*, and since it is a beneficial metabolite present in our body, it is biocompatible, and is likely to have fewer or no side effects. Interventions that increase propionate production in the host, or deliver propionate directly to the host [82] may be applied for *S. aureus* infections. In SSTIs, propionate might be applied to the abscess after incision and drainage [22] to directly control *S. aureus* growth, and therefore decrease excessive inflammation and lead to infection control. Moreover, since resistance against topical antimicrobials has emerged, propionate may be applied as an ointment to a skin wound to prevent infection by *S. aureus*. Furthermore, co-treatment of propionate and a $_{D}$-alanylation inhibitor had a synergistic effect, almost completely inhibiting *in vitro* MRSA growth, and further reduced dermonecrosis *in vivo*. As combination therapy is less prone to resistance, and has been suggested as a way to combat antibiotic-resistant *S. aureus* infections, propionate may be used in combination with other antibiotics that target $_{D}$-alanine motifs, or other bacterial cell wall components. Taken together, these results suggest an alternative strategy using propionate to control antibiotic-resistant *S. aureus* infections (Fig. 22).

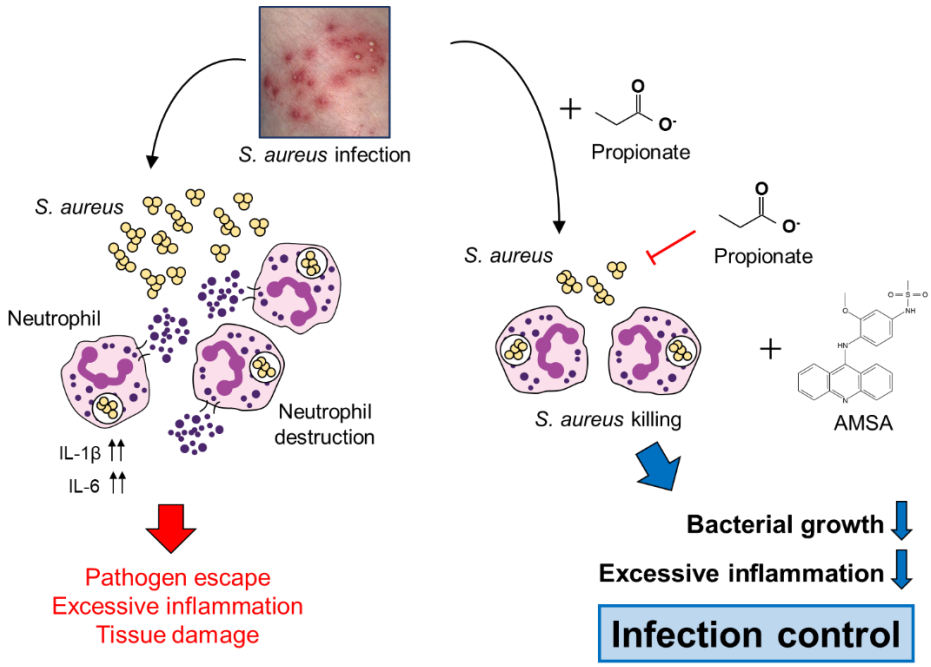

Figure 22. Clinical application of propionate and significance. Upon *S. aureus* skin infection, *S. aureus* will proliferate rapidly, and neutrophils would be recruited. *S. aureus* can evade neutrophil killing and also induce neutrophil lysis. *S. aureus* will escape and cause excessive inflammation and tissue damage. If propionate were used, the growth of *S. aureus* would be inhibited. Excessive inflammation would decrease, and the infection would be controlled. Since propionate is a metabolite and did not cause pathology, it might be used to control *S. aureus* infections without toxicity. Also, combination therapy of propionate with AMSA, a D-alanylation inhibitor, or another cell wall component inhibitor, might be an efficient strategy that is less prone to resistance to control antibiotic-resistant *S. aureus* infections.

Chapter V. References

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국문초록

Propionate에 의한 황색포도상구균 성장 억제 및 감염 제어

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1. 목적

황색포도상구균(*Staphylococcus aureus*)은 그람양성세균으로 피부 및 연조직 감염, 폐렴, 장염과 패혈증 같은 다양한 질병을 유발하는 병원균이다. 특히, 항생제 내성을 가진 *S. aureus*가 증가하면서 치료에 어려움을 겪고 있고, 메티실린 내성을 가진 *S. aureus* (methicillin-resistant *S. aureus*, MRSA)와 다제내성 *S. aureus*에 의한 감염을 제어할 방법이 없기 때문에 문제가 되고 있다. 단쇄지방산은 장내 미생물의 대사물질 중 하나로, 숙주의 장내 건강과 면역 항상성에 중요한 것으로 알려져 있다. 최근에는 단쇄지방산이 미생물에 성장 또는 병독성을 억제할 수 있다는 것이 보고된 바 있다. 하지만 단쇄지방산 acetate, propionate, 또는 butyrate가 황색포도상구균에 미치는 영향은 알려져 있지 않다. 따라서 본 연구에서는 단쇄지방산이 항생제 내성 균주를 포함한 황색포도상구균의 성장과 마우스 피부 감염에 미치는 영향을 알아보았다.

2. 방법

단쇄지방산이 황색포도상구균의 성장에 미치는 영향을 알아보기 위해 여러 농도의 acetate, propionate, 또는 butyrate를 처리한 후 분광광도계로 흡광도를 측정하여 성장을 측정하였다. 단쇄지방산이 정균적 또는 살균적 효과를 가지는지 보기위해 minimum inhibitory concentration/minimum bactericidal concentration 테스트를 진행하였다. 황색포도상구균의 형태를 주사전자현미경을 통해 확인하였다. 또한, 단쇄지방산이 MRSA 피부감염에 미치는 영향을 알아보기 위해 마우스에 MRSA 또는 MRSA와 단쇄지방산을 피하주사로 감염시키고 형성된 농양의 크기와 무게를 측정하였다. 농양을 균질화 하여 MRSA의 양을 측정하였고, 상층액에서 interleukin (IL)-1β와 IL-6를 효소결합 면역분석법을 통해 측정하였다. 농양을 동결절편을 하여 hematoxylin & eosin 염색과 그람 염색을 통해 조직학적 분석을 하였다. 황색포도상구균의 세포벽 물질과 단쇄지방산에 의한 성장 억제의 관련성을 알아보기 위해 lipoteichoic acid (LTA), wall teichoic acid (WTA), lipoprotein, 또는 teichoic acid의 $_{D}$-alanine분자가 결손 된 황색포도상구균을 사용하여 야생형 균주와 비교하였다. 또한, MRSA에 $_{D}$-alanylation 억제제를 이용하여 성장을 측정하고 단쇄지방산과의 병용처리의 효과를 확인하였다. 단쇄지방산의 작용기전을 알아보기 위해 대사에 관련된 효소가 결손 된 MRSA를 사용하여 성장을 측정하였다. 단쇄지방산이 다른 세균에 미치는 영향 또한 확인하였다.

3. 결과

단쇄지방산 중 propionate가 가장 효과적으로 MRSA의 성장을 억제하였으며 propionate는 실제 환자에게서 분리 된 균주와 다제내성 균주를 포함한 모든 황색포도상구균의 성장을 억제하였다. Propionate는 MRSA 피부감염에서 농양 형성, MRSA의 수 및 과도한 IL-1β와 IL-6 생성을 억제하였다. 또한, propionate는 MRSA 감염이 시작된 후에 처리되었을 때에도 농양 형성을 감소시켰다. LTA 또는 WTA가 결손 된 경우 단쇄지방산에

의해 성장이 더 억제 되었고, teichoic acid에 공통적으로 존재하는 $_{D}$-alanine이 결손 된 경우 propionate에 의한 성장억제에 더 민감하였다. $_{D}$-Alanylation 억제제를 처리한 MRSA 또한 propionate에 의해 성장이 더 억제 되었고, $_{D}$-alanylation 억제제와 propionate를 병용처리 하였을 때 농양 형성, MRSA의 수와 과도한 싸이토카인 생성이 억제되어 MRSA 피부감염이 더욱 완화되는 것을 확인하였다. 해당과정의 효소가 결손 된 MRSA는 propionate에 의해 비슷한 정도로 억제 되었지만, 포도당신생합성의 효소가 결손 된 MRSA는 성장이 더 많이 억제 되었다. Tricarboxylic acid 회로 효소가 결손 된 경우 또한 성장이 더 많이 억제 되어 propionate와 대사의 관계성을 확인하였다. Propionate는 황색포도상구균 뿐 아니라 다른 병원균, 폐렴구균(*Streptococcus pneumoniae*)과 장구균 *Enterococcus faecium*의 성장을 억제하였고, 공생균 표피포도상구균 (*Staphylococcus epidermidis*), 장구균 *Enterococcus faecalis*의 성장을 약간 억제하였으나 공생균 *Streptococcus gordonii*와 유산균 *Lactobacillus plantarum*의 성장은 억제하지 않았다.

4. 결론

본 연구에서는 propionate가 다제내성 균주 및 실제 환자에서 분리된 균주를 포함한 여러 황색포도상구균의 성장을 억제하였고 MRSA 피부감염을 완화시켰다. Propionate와 $_{D}$-alanylation 억제제를 병용처리 한다면 다제내성 황색포도상구균 감염을 제어할 수 있을 것이라고 예상된다.

주요어: 황색포도상구균, MRSA, Propionate, 단쇄지방산, $_{D}$-Alanine
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