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Propionic acid and its esterified derivative suppress the growth of methicillin-resistant *Staphylococcus aureus* USA300

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RESEARCH ARTICLE

Abstract

Previously, we demonstrated that *Propionibacterium acnes*, a human skin commensal bacterium, ferments glycerol into short-chain fatty acids, including propionic acid. Propionic acid suppressed the growth of *Staphylococcus aureus* USA300, a community-acquired methicillin-resistant bacterium, *in vitro* and *in vivo*. In this study, it is demonstrated that the anti-USA300 activity of propionic acid persisted after buffering the acid with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. This suggests that the growth suppression of USA300 mainly resulted from the antimicrobial activity of propionic acid per se and not from the acidity of the medium. In addition, proprionic acid significantly reduced the intracellular pH of USA300 and exhibited broad-spectrum antimicrobial activity against *Escherichia coli* and *Candida albicans*. *P. acnes* showed a higher tolerance to propionic acid. Next, an esterified derivative of propionic acid was synthesised. Propionic acid and the esterified derivative were equivalent in their efficacy to suppress the growth of USA300 *in vitro*. The esterified derivative thus provides an alternative to propionic acid as an antimicrobial agent against *S. aureus.*

Keywords: fermentation, *Propionibacterium acnes*, probiotics, propionic acid, *Staphylococcus aureus*

1. Introduction

Propionibacterium acnes, a skin commensal bacterium, gets its name from its ability to produce propionic acid, a short-chain fatty acid (SCFA), during fermentation (Higaki *et al.*, 2004; Ushijima *et al.*, 1984). A number of SCFAs are commonly found in the skin and in the secretion of skin glands, such as sweat (Burtenshaw, 1942). SCFAs affect a range of host processes, including host-microbe signalling and control of pH, with subsequent effects on the composition of the microbiome (Nicholson *et al.*, 2012). Several SCFAs have been approved by the US Environmental Protection Agency as active ingredients for use as fungicides and bactericides on stored grains, poultry litter, and drinking water for poultry and livestock (Sebastian *et al.*, 1996). It is known that propionic acid has antimicrobial activity (Cherrington *et al.*, 1991). As a food additive, propionic acid is listed as 'generally recognized as safe' by the US Food and Drug Administration (FDA). A previous study revealed that *P. acnes* is a probiotic bacterium and can convert glycerol into propionic acid by fermentation in mouse skin (Shu *et al.*, 2013). Propionic acid effectively suppressed the growth of *Staphylococcus aureus* USA300, a community-acquired methicillin-resistant (CA-MRSA) bacterium, reported as the most common cause of purulent skin infections in the USA (Kaplan *et al.*, 2005; Lutmer *et al.*, 2013). The mechanism of action of propionic acid against *S. aureus* and the broad-spectrum antimicrobial activity of propionic acid against other pathogens have not yet been evaluated.

Everyone hosts *P. acnes* (Ahn *et al.*, 1996; Grice and Segre, 2011), which comprises around 50% of the total skin microbiome, a diverse milieu of microorganisms with an estimated density of 10^2 to 10^6 bacteria per cm^2 on the skin surface (Evans, 1975; Evans *et al.*, 1984; Ramstad *et al.*, 1997). *P. acnes* also is a regular resident within the hair follicle (Zouboulis, 2004). Glycerol, a carbon source for bacterial fermentation, is a naturally occurring metabolite and humectant found in the human skin (Fluhr *et al.*, 2008). It has been reported that glycerol can be produced endogenously by breakdown of triglycerides by sebaceous gland-associated lipase in the human skin (Smith and Thiboutot, 2008). A previous study showed that *P. acnes* was present in wounds after dermatologic surgery (Saleh *et al.*, 2011). Thus, it is possible that *P. acnes* can migrate into a deep wound from the surface of the wounded skin and/or ruptured hair follicles. The anaerobic microenvironment in deep-seated wounds may initiate the process of fermentation by *P. acnes* metabolising glycerol into propionic acid, which kills pathogens and prevents the entry of pathogens into the bloodstream.

S. aureus is responsible for a broad range of infections, from cellulitis to disseminated systemic infections, leading to organ failure and death (Al Mohajer *et al.*, 2013; Malani *et al.*, 2008). Although incision and drainage procedures are performed for the majority of patients with *S. aureus* abscesses of the skin, antimicrobials are usually given topically or systemically as adjunctive therapy to maximise the chance of clearing the pathogen. Despite the fact that several SCFAs have been approved by the FDA for use in foods as bactericides (Collins, 1971; Pradhan *et al.*, 2009), they are not among the antimicrobials used for treating infections in humans. The major problem with the use of SCFAs as antimicrobials has been to achieve and maintain millimolar concentrations *in vivo*. SCFAs can be metabolized rapidly as soon as they enter cells via the active transport system (Schröder *et al.*, 2000; Stein *et al.*, 1995, 2000). To overcome this pharmacokinetic drawback, we have synthesized a propionic acid derivative containing two propionic acid moieties esterified to a chemical linker to retain the antimicrobial properties of propionic acid.

In this study, we demonstrated that propionic acid markedly reduced the intracellular pH of *S. aureus* USA300 and exhibited broad-spectrum antimicrobial activity against *Escherichia coli* and *Candida albicans*. Both propionic acid and its esterified derivative efficiently suppressed the growth of USA300. Propionic acid, produced by fermentation of glycerol by commensal *P. acnes*, might naturally occur in bacteria-infected skin wounds and be part of skin immunity against pathogens. The esterified derivative of propionic acid, designed on the basis of a natural strategy (fermentation by *P. acnes*) shows promise as a novel antimicrobial to combat pathogens.

2. Materials and methods

Microorganisms

P. acnes (ATCC 6919; American Type Culture Collection, Manassas, VA, USA) was cultured on Reinforced Clostridial Medium (Oxoid, Basingstoke, UK) under anaerobic conditions using Gas-Pak (BD, Sparks, MD, USA) at 37 °C. *S. aureus* (USA300) was cultured on 3% tryptic soy broth (TSB) Sigma, St. Louis, MO, USA) agar overnight at 37 °C. *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) was cultured on Luria broth agar (Difco; BD) at 26 °C for 48 to 72 h. *C. albicans* (ATCC14053) was grown in an orbital incubator at 30 °C in 3% Sabouraud dextrose broth (Sigma) overnight. All microorganisms were cultured from a single colony. Overnight cultures were diluted 1:100 and cultured until they reached an optical density at 600 nm (OD_{600}) of approximately 1.0. Microorganisms were harvested by centrifugation at 5,000×*g* for 10 min, washed with phosphate buffered saline (PBS) (pH 7.4), and suspended in an appropriate amount of PBS for further experiments.

Minimal bactericidal and fungicidal concentration tests

To determine the minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) of propionic acid, microorganisms (10^6 colony forming units (cfu)/ml) were incubated overnight in media with propionic acid (1.25-2,000 mM) in a 96-well microplate (100 µl per well). The control only received PBS. After incubation, the microorganisms were diluted 1:10-1:10^6 into PBS. The dilutions (5 µl) were spotted onto agar media to count cfu; MBC/MFC were determined at 99.9% killing level. To determine the effect of pH on growth of *S. aureus* USA300, the bacterium was incubated in TSB at pH 5.8 and 6.8, and TSB with 25 mM propionic acid at pH 5.8 and 6.8 (buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) in a 96-well microplate overnight before spotting onto agar plates.

Radial diffusion assay

A radial diffusion assay (RDA) was performed as previously described with minor modifications (Chen *et al.*, 2011). Briefly, microorganisms in the mid-log phase were centrifuged at 12,000×*g* for 10 min, washed with PBS, and dispersed (10^5 cfu/ml) in agar consisting of 1% (w/v) agarose (Sigma) and 3% (w/v) culture medium in PBS at 42 °C. Then, the agar was poured into Petri dishes and solidified. Wells (diameter 3 mm and volume 30 µl) were created by poking a pipette tip into the semi-solidified agar. Propionic acid was serially diluted in PBS to concentrations ranging from 5 to 2,000 mM, and 30 µl aliquots were added to the wells; PBS served as the control. After 3 h of incubation, a 10 ml overlay gel composed of 3% culture medium and 1% agarose was poured onto the plates, whereafter the

plates were incubated overnight and examined for zones of growth inhibition.

Intracellular pH

S. aureus USA300 bacteria were loaded with 5 μM carboxyfluorescein succinimidyl ester (cFSE) (Life Technologies, Grand Island, NY, USA) for 30 min at 37 °C in 50 mM HEPES and 5 mM ethylenediamine tetraacetic acid (EDTA) as previously described (Chitarra *et al.*, 2000). To remove unbound probe, bacteria were incubated with glucose (10 mM) for an additional 30 min, washed twice in PBS with 10 mM $MgCl_2$ and resuspended in PBS. cFSE-loaded USA300 (3×10^4 cfu) were dispensed in a 96-well microplate (100 μl per well) containing 25 mM propionic acid or PBS. Fluorescence was measured for 5 min every min using an excitation wavelength of 490 nm and an emission wavelength of 520 nm. A reduction in relative fluorescence reflected a decrease in intracellular pH. Fluorescence of bacteria-free supernatant obtained by centrifugation at 5,000×*g* for 5 min after the 5-min assay was measured to correct for background fluorescence. Calibration curves were obtained by incubation of untreated, cFSE-loaded bacteria in buffer containing 50 mM glycine, 50 mM cittric acid, 50 mN $Na_2HPO_4.2H_2O$ and 50 mM KCl adjusted to various pH values from 4-10. 1 μM valinomycin and nigericin (Sigma) were added to equilibrate the intracellular and extracellular pH.

Synthesis of propionic acid 2-(2-propionyloxyethoxy) ethyl ester

50 mmol propionic acid and 20 mmol diethylene glycol (DEG) in 100 ml dichloromethane were added to 60 mmol *N,N'*-dicyclohexyl carbodimide portionwise. The cloudy white suspensions were stirred at room temperature overnight, then filtered and washed with hexane. The filtrate was concentrated under reduced pressure to yield pure and colourless propionic acid 2-(2-propionyloxyethoxy)ethyl ester (PA-DEG-PA; >97%, 2.3 g), which was purified by chromatography (silica gel) eluted with 10% ethyl ethanoate/hexane. PA-DEG-PA was validated by 1H NMR (300 MHz) analysis (Avance DPX-300; Bruker, Fremont, CA, USA) using chloroform solvent. Signals [δ 4.23 (m, 2H), 3.70 (m, 2H), 2.36 (J=8 Hz, 2H), 1.14 (J=8 Hz, 3H)] were detected in NMR spectroscopy.

Statistical analysis

All statistical tests were performed using a two-tailed t-test. *P*-values <0.05 were considered statistically significant.

3. Results

Effect of propionic acid on growth of USA300

In a previous study, acetic acid, lactic acid and propionic acid were detected as products of glycerol fermentation by *P. acnes* in a 2-D ^1H-^{13}C heteronuclear single quantum correlation NMR spectrum (Shu *et al.*, 2013). This demonstrated the ability of *P. acnes* to ferment glycerol. It has been reported that propionic acid is a unique end-product of glycerol fermentation in *Propionibacterium* species compared with other fermenting bacteria (Dishisha *et al.*, 2012). Propionibacteria can inhibit the growth of a number of microorganisms due to their production of propionic acid (Lind *et al.*, 2005). Therefore, propionic acid was selected for evaluation of its antimicrobial activity. As *S. aureus* USA300 is a leading bacterial pathogen in both hospital and community settings in the USA and many other countries (Stefani *et al.*, 2012), it was selected as a model pathogen for evaluation of the antimicrobial activity of propionic acid. In accordance with our previous study (Shu *et al.*, 2013), results from the MBC tests showed that propionic acid efficiently inhibited growth of USA300 more than 1 $\log_{10}$ reduction at concentrations greater than 25 mM, and completely killed the bacterium at concentrations greater than or equal to 100 mM (data not shown). In addition, consistent with the results of the MBC tests, growth inhibition zones in radial diffusion assays were also clearly observed when USA300 was incubated with propionic acid at concentrations greater than 25 mM (data not shown).

Effect of pH on activity of propionic acid against USA300

The pH dropped from 6.8 to 5.8 when mM propionic acid at a concentration of 25 mM, which corresponded to the MBC, was added to a culture of USA300 in TSB. To validate that the growth suppression of USA300 by propionic acid was not due to the acidity of the medium, the bacterium was incubated in TSB at pH 5.8 and 6.8, and TSB with 25 mM propionic acid at pH 5.8 and 6.8 (buffered with HEPES). The bacterial numbers after overnight incubation are shown in Figure 1A. Buffered propionic acid resulted in some decrease in cfu/ml (>1 log10) compared to TSB at pH 5.8 and 6.8. However, these results suggested that growth suppression of USA300 mainly resulted from the antimicrobial activity of propionic acid and not from the acidity of the medium.

Effect of propionic acid on intracellular pH of USA300

It has been documented that the undissociated form of SCFAs significantly contributes to their antimicrobial effect (Ostling and Lindgren, 1993; Ricke, 2003). Undissociated SCFAs can passively diffuse through the bacterial cell wall, dissociate, and reduce the neutral pH of the cytoplasm,

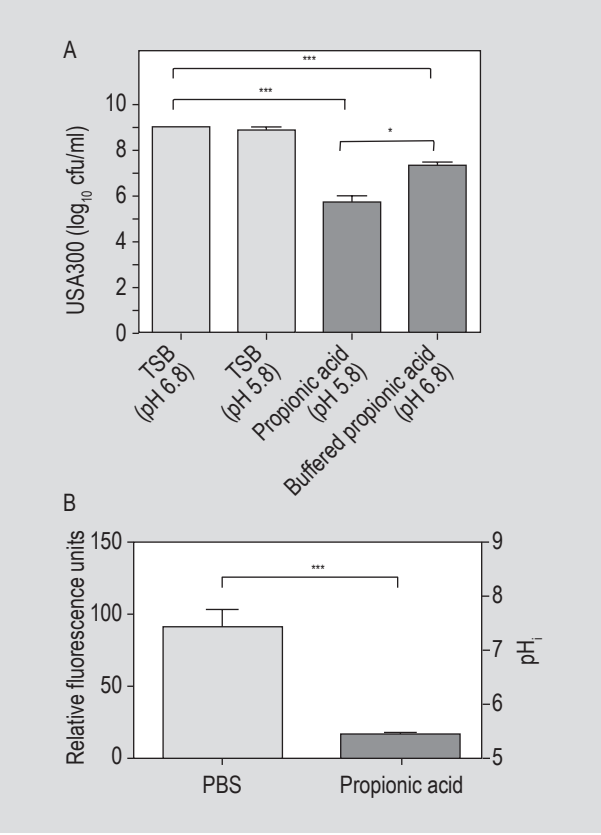

Figure 1. (A) Effect of pH on activity of propionic acid against *Staphylococcus aureus* USA300 and (B) effect of propionic acid on intracellular pH (pH_i) of this bacterium. PBS = phosphate buffered saline; TSB=tryptic soy broth. Data are the mean ± standard deviation of three individual experiments. *P<0.05; ***P<0.001.

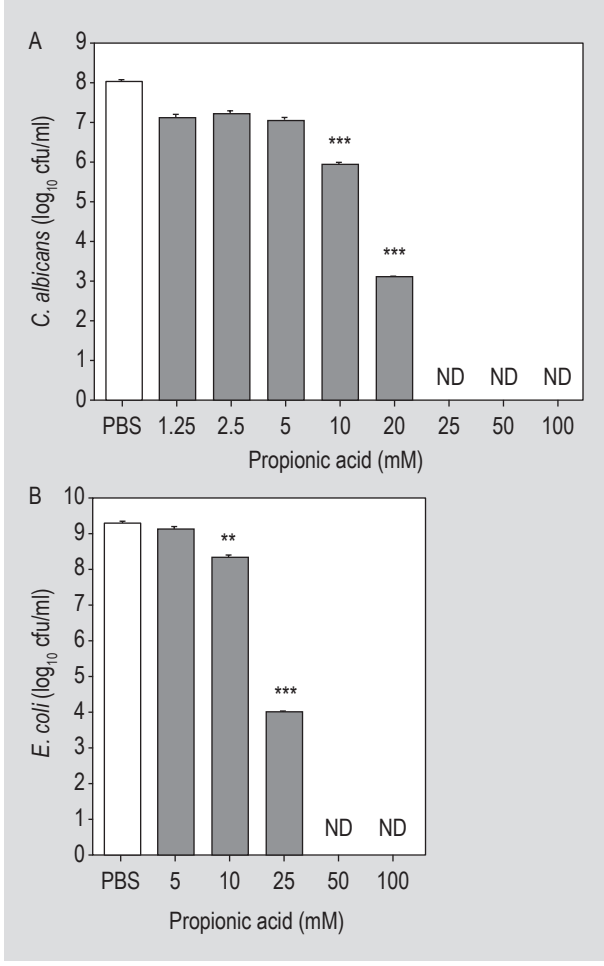

Figure 2. (A) Minimal fungicidal concentration of propionic acid against *Candida albicans* and (B) minimimal bactericidal concentration against *Escherichia coli*. ND = not detectable; PBS = phosphate buffered saline. Data are the mean ± standard deviation of three individual experiments. **P<0.01; ***P<0.001.

eventually leading to bacterial lysis. To explore the mechanism of action of propionic acid on *S. aureus* USA300, the bacteria were loaded with cFSE, an internally conjugated fluorescent probe, to determine intracellular pH. As shown in Figure 1B, propionic acid, but not PBS, considerably lowered the intracellular pH of USA300, supporting the previous findings that a reduction in intracellular pH is the lethal mechanism of propionic acid.

Effect of propionic acid on growth of *Escherichia coli* and *Candida albicans*

To examine if propionic acid exerts broad-spectrum antimicrobial activity, we determined its effect on *C. albicans*, a fungus that can cause superficial infections of skin and mucosal membranes, and *E. coli*, a Gram-negative bacterium that can be frequently isolated from human skin. These pathogens were incubated with propionic acid at various concentrations in MBC tests. It was found that propionic acid effectively suppressed the growth of *C. albicans* and *E. coli* (Figure 2) at propionic acid concentrations greater than 10 mM, and completely killed them at concentrations greater than or equal to 25 and 50 mM, respectively. Consistent with the results of the MBC tests, growth inhibition zones in radial diffusion assays were observed when these pathogens were incubated with propionic acid at a minimum effective concentration of 10 mM (data not shown). Taken together, these findings suggest that propionic acid displays broad-spectrum antimicrobial activity.

Effect of propionic acid on *Propionibacterium acnes*

To investigate if propionic acid exhibits antimicrobial activity against *P. acnes*, this bacterium was incubated with propionic acid at concentrations ranging from 5 to 2,000 mM in MBC tests and radial diffusion assays. As shown in Figure 3, propionic acid at concentrations between 5 and 500 mM did not affect the growth of *P. acnes* in MBC tests. Growth inhibition was only detectable concentrations greater than 1000 mM, indicating that *P. acnes* has a high

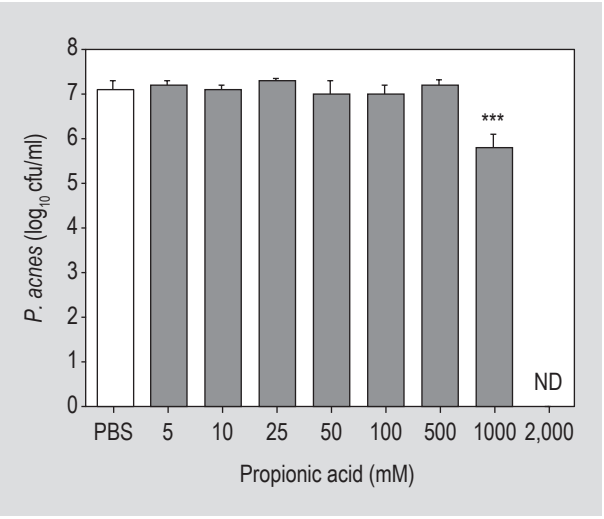

Figure 3. Minimal bactericidal concentration of propionic acid against *Propionibacterium acnes*. ND = not detectable; PBS = phosphate buffered saline. Data are the mean ± standard deviation of three individual experiments. ***$P<0.001$.

tolerance to propionic acid, which is produced by this bacterium during fermentation. Radial diffusion assays were consistent with these results (data not shown). A previous study demonstrated that *S. aureus* showed higher sensitivity to propionic acid than *Staphylococcus epidermidis*, a Gram-positive bacterium predominately found on human skin (Hellmark *et al.*, 2013; Ushijima *et al.*, 1984). These findings suggest that the risk of propionic acid as a component of a skin probiotic to suppress the growth of dominant skin bacteria, such as *P. acnes* and *S. epidermidis*, is lower.

Effect of esterified derivative of propionic acid on growth of USA300

SCFAs produced by intestinal microbes in the human colon can reach a high level (20-140 mM) that can effectively kill local pathogens, whereas their concentration in peripheral circulation is generally low (3-7 μM) (Garland, 2011). A number of SCFAs are commonly found in the skin and in the secretions of skin glands, such as sweat, but their concentrations are relatively low, for example 0.0062% for propionic acid in sweat (Burtenshaw, 1942). Although the levels of SCFAs in skin lesions have yet to be determined, it has been reported that SCFAs have short half-lives and thus achieving pharmacologic concentrations *in vivo* is apparently difficult. Several esterified derivatives of SCFAs, e.g. pivaloxylomethyl butyrate (AN-9) as an esterified butyric acid (Hobdy and Murren, 2004), have been developed to achieve effective concentrations of SCFAs. In the present study, we synthesized PA-DEG-PA, an esterified derivative of propionic acid that contains two active propionic acids esterified to a DEG linker (Figure 4A and B). MBC tests were conducted to assess its anti-*S. aureus* USA300 activity (Figure 4B). To compare the effect of PA-DEG-PA with propionic acid, USA300 was incubated overnight with the same concentrations (0-100 mM) PA-DEG-PA or propionic acid, dissolved in 4% dimethyl sufoxide. Similar to previous results (Shu *et al.*, 2013), the

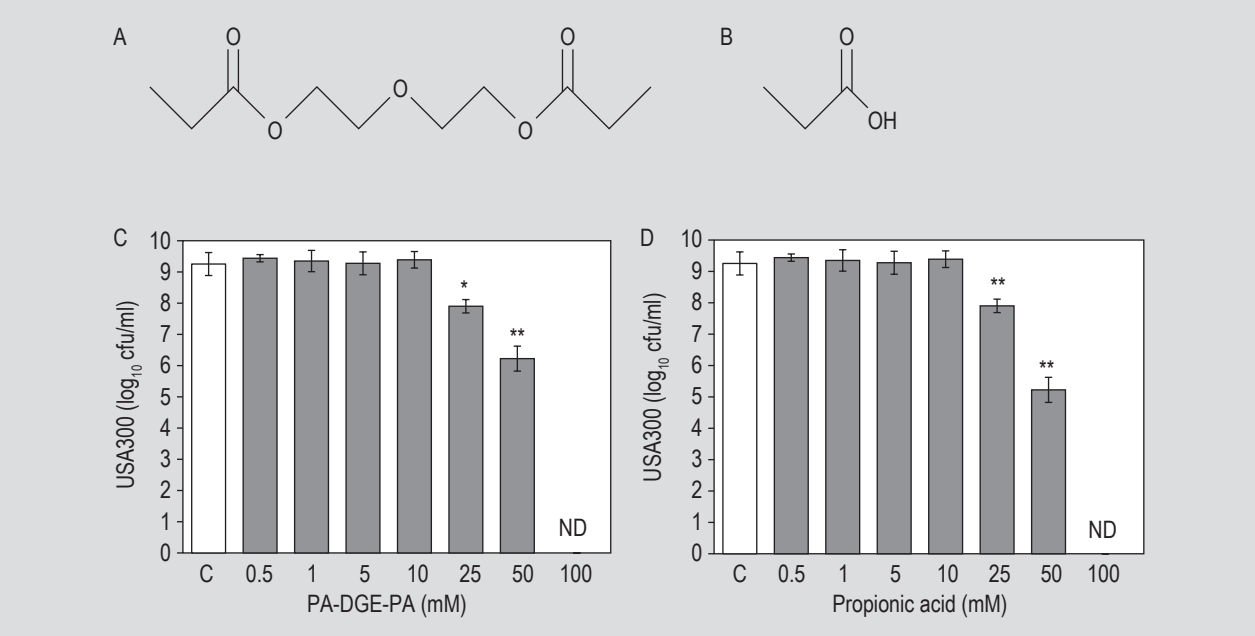

Figure 4. (A) Chemical structure of propionic acid 2-(2-propionyloxyethoxy)ethyl ester (PA-DEG-PA) composed of two propionic acid moieties and (B) esterified to a DEG linker; (C) and (D) minimum bactericidal concentration of PA-DEG-PA and propionic acid against *Staphylococcus aureus* USA300. ND = undetectable; C = control (4% dimethyl sufoxide). Data are the mean ± standard deviation of three individual experiments. *$P<0.05$; **$P<0.01$.

MBC (>1 $\log_{10}$ inhibition) of propionic acid was 25 mM and the concentration for growth complete inhibition was 100 Mm (Figure 4B). PA-DEG-PA also inhibited growth of USA300 by more than 1 $\log_{10}$ at a concentration ≥25 mM; growth inhibition was complete at 100 mM (Figure 4B), suggesting that PA-DEG-PA was equivalent in efficacy to suppress the growth of USA300 *in vitro*.

4. Discussion

MRSA is responsible for more than 94,000 serious infections and nearly 19,000 deaths per year in the USA (Boe *et al.*, 1964). The bacteria remain one of the most important causes of nosocomial infections worldwide (Chatterjee and Otto, 2013). The skin and soft tissues are the most common sites of *S. aureus* infection and comprise more than 75% of MRSA disease (Cohen *et al.*, 2007). Conditions that help spread MRSA include close skin-to-skin contact, sharing personal hygiene articles, and wounds in the skin. Antibiotics are commonly used to curtail MRSA infections. However, the use of non-endogenous antibiotics for treatment is not in compliance with evolutionary medicine, as bacteria may develop the ability to neutralize these antibiotics.

Propionic acid is an endogenous molecule in human skin (Burtenshaw, 1942). It is known that endogenous bactericides are largely non-specific and hold great promise to avert the development of bacterial resistance (Gibbons *et al.*, 2006; Peschel and Sahl, 2006; Smith and Romesberg, 2007). It has been proposed that endogenous bactericides and innate bactericide resistance mechanisms have co-evolved, leading to a transient host-pathogen balance that has shaped the existing repertoire of endogenous bactericides (Peschel and Sahl, 2006). There are only a few topical medications that are used for prevention of *S. aureus* infection in skin wounds. In a previous study, we demonstrated that pretreatment of skin wounds with propionic acid effectively mitigated subsequent infection with CA-MRSA in mice (Shu *et al.*, 2013). A recent study showed that the probiotic *Lactobacillus reuteri* protected normal human epidermal keratinocytes from *S. aureus* infection *in vitro* (Prince *et al.*, 2012). In addition, culture supernatants and live *Lactobacillus plantarum* cells, isolated from vinegar, and applied to transcutaneous wounds on the backs of mice infected with *S. aureus*, isolated from several wound infection specimens, succeeded in preventing *S. aureus* from establishing wound infection (Al-Mathkury and Al-Aubeidi, 2008). These studies clearly demonstrate that fermenting bacteria and their fermented products can function as probiotics against *S. aureus* (Sikorska and Smoragiewicz, 2013). However, both *L. reuteri* and *L. plantarum* do not belong to skin commensal bacteria. Application of such bacteria onto the skin may disrupt the ecosystem balance of the human skin microbiome (Mathieu *et al.*, 2013).

Skin commensal bacteria may enter deep wounds counteract pathogens by their fermentation products. *P. acnes* can be found in wounds (Bowler *et al.*, 2001) and deep tissue infection (Aleissa *et al.*, 2011) in humans. In addition, SCFAs produced by bacterial fermentation have been detected in deep-seated abscesses, anaerobic microenvironments in the context of human bacterial infection (Demaerel *et al.*, 1994). Although there is no direct evidence that SCFAs produced by fermenting *P. acnes* can suppress the growth of pathogens in deep-seated abscesses in humans, results from a previous study demonstrated that *P. acnes* can ferment glycerol fermentation in skin wounds and diminish the colonization of CA-MRSA in mice (Shu *et al.*, 2013). Propionic acid, a major SCFA produced by *P. acnes* fermentation, inhibited the growth of CA-MRSA *in vitro* and *in vivo* (Shu *et al.*, 2013), while having less effect on *P. acnes* itself. It has been reported that *P. acnes* co-existed with *S. aureus* in shoulder sepsis (Bashir *et al.*, 2007) and prosthetic hip infections (Ramage *et al.*, 2003) in adult patients. However, the role of *P. acnes* as a probiotic or harmful bacterium in these infections remains unsolved. MBCs/MFCs of propionic acid for USA300, *C. albicans* and *E. coli* were 25, 10, and 10 mM, respectively, indicating that *C. albicans* and *E. coli* are more susceptible to propionic acid than USA300.

Skin probiotics based on skin commensal fermenting bacteria are not yet developed. The World Health Organization (WHO) defines probiotics as 'live micro-organisms that confer a health benefit to the host and are generally regarded as safe in humans' (Licciardi *et al.*, 2012). *P. acnes* can modulate antimicrobial peptide and chemokine expression of skin cells via Toll-like receptor 2 (Nagy *et al.*, 2005). SCFAs have been recognized as ligands for free fatty acid receptor 1 (also named G protein-coupled receptor 40) (Hara *et al.*, 2011). Previous studies showed that an arylalkyl derivative of propionic acid, reduced chemokine induction and immune inflammation in skin cells via activation of the FFAR1 receptor (Fujita *et al.*, 2011). In the present study, we synthesised an esterified derivative of propionic acid, PA-DEG-PA, that was equivalent in efficacy to propionic acid in inhibiting growth of USA300. Theoretically, PA-DEG-PA can be cleaved by esterases to release two propionic acids. Both *S. aureus* (Holden *et al.*, 2010) and skin cells (Batz *et al.*, 2013) express esterases. Future studies will include determining which active compounds (PA-DEG-PA, released propionic acid or both) contribute to the activity of PA-DEG-PA against *S. aureus*. Propionic acid at 100 mM can significantly eliminate the colonization of USA300 in mouse skin (Shu *et al.*, 2013). It is worth investigating if PA-DEG-PA has a longer half-life than propionic acid in the skin and can ward off *S. aureus* skin infection at a lower dose.

5. Conclusions

The skin commensal *P. acnes* can produce propionic acid by glycerol fermentation (Shu *et al.*, 2013). Propionic acid inhibited the growth of *S. aureus* USA300 by a decrease in intracellular pH; it also exerted antimicrobial activities against *C. albicans* and *E. coli*. An esterified derivative of propionic acid was synthesized and showed equivalent efficacy to propionic acid in inhibiting the growth of *S. aureus* USA300 *in vitro*.

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