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Synergistic activity of weak organic acids against uropathogens

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Running title: Organic acid synergy against uropathogens

Summary

Background

Urinary tract infections (UTIs) are among the most common hospital-acquired infections, with an estimated 75% of UTIs caused by urinary catheters. In addition to the significant healthcare costs and patient morbidity, the escalating antimicrobial resistance reported among common uropathogens make the investigation of efficacious new antimicrobial strategies of urgent importance.

Aim

To examine the antibacterial activity of a suite of weak organic acids (WOAs) (citric acid, malic acid, propionic acid, mandelic acid, lactic acid, benzoic acid, pyruvic acid and hippuric acid), alone and in combination, against common nosocomial uropathogens (*Proteus mirabilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*).

Methods

Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum biofilm eradication concentration (MBEC), fractional inhibitory concentration index

(FICI) values and kinetics of bactericidal activity of WOAs were determined by microdilution and time-kill assays.

Findings

All tested WOAs displayed bactericidal activities against uropathogens in their planktonic and biofilm modes of growth when used individually. Moreover, WOAs in combination displayed synergistic activity against *P. mirabilis*, *S. aureus* and *E. coli*, with reductions in MIC values of up to 250-fold and significant reductions in biofilm formation.

Conclusion

The synergistic multi-mechanistic combinations identified herein are anticipated to play an important role in the treatment and prevention of catheter-associated UTIs.

Keywords: Uropathogen; antimicrobial resistance; synergy; weak organic acid; biofilm; urinary catheter

Introduction

Urinary catheters are the most commonly employed medical device, with a reported 15 - 25% of hospitalised patients undergoing catheterisation [1-3]. These devices are, however, highly susceptible to infection. Their position in the bladder creates optimal conditions for bacterial biofilm growth and, furthermore, disrupts the bladder's normal mechanism of defence by which it flushes out contaminating bacteria [4]. Furthermore, urinary catheters are implicated in up to 75% of hospital-acquired UTIs and direct treatment costs of catheter-associated urinary tract infections (CAUTIs) exceed \$350 million annually [2, 5]. If left without treatment, infections can develop into pyelonephritis, septicaemia and shock [6]. Moreover, ~50% of long-term catheterised patients experience recurrent catheter blockages due to encrustation [7]. The main causative uropathogens are *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis* [1]. The World Health Organisation (WHO) has reported rising resistance of common nosocomial pathogens, including many bacteria commonly isolated from CAUTIs, to antibiotics, making treatment particularly challenging [2].

There has therefore been much interest in efficacious new strategies to prevent infections without necessitating the use of antibiotics, for example by modification of the device itself through the application of antibacterial-containing catheter coatings [8]. Examples of currently investigated agents include chlorhexidine, silver ions and triclosan. Issues associated with toxicity, suboptimal release profiles and limited efficacy *in vivo* have, however, restricted their widespread clinical application [2]. More recently, the use of combinations of antibacterial agents has attracted interest due to the potential for increasing antimicrobial efficacy and broadening the spectrum of activity, thereby leading to pathogen eradication with reduced development of resistance [9]. Furthermore, the use of antibacterial agents in combination is anticipated to reduce the corresponding concentrations required, thus avoiding toxicity issues [10].

Combinations of weak organic acids (WOAs), for example malic and citric acid, have been demonstrated to reduce colonisation of the bladder by uropathogenic *E. coli* in an experimental mouse model of ascending UTI, while mixtures of mandelic and lactic acid have demonstrated activity against *E. coli* cells in both their planktonic and biofilm modes of growth [11, 12]. WOAs have been used as naturally occurring preservatives in food and beverages for centuries and, importantly, their efficacy against antibiotic-resistant bacteria has recently been demonstrated [13]. Kundukad *et al.* (2020) investigated the antibacterial activity of citric acid

against a broad range of antibiotic-resistant strains of *Klebsiella pneumoniae*, *P. aeruginosa* and *S. aureus*, and confirmed the ability of this triprotic acid to eradicate cells in their planktonic and biofilm modes of growth [14]. These agents are reported to exert their antimicrobial effect by the flow of undissociated molecules through the bacterial cell membrane by diffusion, disrupting the transmembrane proton motive force [13]. The resulting decrease in intracellular pH causes damage to enzymes, proteins and DNA structure, and inhibits metabolic processes [15]. Furthermore, the accumulation of released anions may have an osmotic effect and increase turgor pressure [14]. In addition to the pH-lowering effect, Alakomi *et al.* (2000) reported permeabilization of Gram-negative bacterial membranes, with subsequent release of lipopolysaccharides, by lactic acid. Destabilisation of outer membranes of *Salmonella* by citric and malic acid due to cation chelation and/or intercalation has also been demonstrated [16-18].

In light of their long history of use as antibacterial and antifungal agents in the food industry, their low cost, wide availability and the absence of high levels of resistance promotion, WOAs constitute attractive agents to minimise unnecessary antibiotic use [19]. Herein we investigate the antimicrobial activity of a suite of WOAs, alone and in combination, to inform the potential use of these agents in the prevention and treatment of the global healthcare priority of CAUTIs.

Materials

Citric acid, malic acid, propionic acid, mandelic acid, lactic acid, benzoic acid, pyruvic acid, hippuric acid, benzalkonium chloride (BKZ), L-histidine ($\geq 98.5\%$), L-cysteine ($\geq 97\%$), reduced glutathione ($\geq 98\%$), tris(hydroxymethyl)aminomethane hydrochloride and sodium chloride were purchased from Sigma Aldrich (Poole, Dorset, UK). All formulated bacteriological media were obtained from Oxoid Ltd. (Hampshire, UK). *P. mirabilis* ATCC 51286 and *S. aureus* ATCC 29213 were purchased from LGC Standards (Middlesex, UK). Clinical isolates of *E. coli* NSM59 and *P. aeruginosa* 679 were obtained from Dr. Brian Jones, University of Bath (UK). Bacterial strains were maintained on cryotubes in 15% glycerol at -80°C . Live/Dead BacLight™ bacterial viability kit (L7012) was purchased from Thermo Fisher Scientific (Dublin, Ireland).

Methods

Preparation of WOA stock solutions

Stock solutions were prepared in Mueller–Hinton broth (MHB) at the following concentrations: citric acid (96 mg mL⁻¹), malic acid (67 mg mL⁻¹), propionic acid (11 mg mL⁻¹), mandelic acid (76 mg mL⁻¹), lactic acid (45 mg mL⁻¹), pyruvic acid (44 mg mL⁻¹), benzoic acid (7 mg mL⁻¹) and hippuric acid (8.3 mg mL⁻¹). A stock solution of the antiseptic BKZ (0.2 mg mL⁻¹) was also prepared in MHB. The solutions were then sterilised by syringe filtration with a 0.20 µm filter and stored at -80°C.

Neutraliser preparation

Universal neutraliser of L-histidine, L-cysteine and reduced glutathione was prepared to neutralise the effects of residual acids in the microbiological tests. A solution of 1.0 g L-histidine, 1.0 g L-cysteine and 2.0 g reduced glutathione was made up to 20 mL in dH₂O and then sterilised by syringe filtration with a 0.20 µm filter. 500 µL of the universal neutraliser solution was added to each 20 mL of medium used to give respective concentrations of 0.125% w/v L-histidine, 0.125% w/v L-cysteine and 0.25% w/v reduced glutathione [20].

Determination of antimicrobial activity

Minimum inhibitory concentration (MIC) values of WOAs against *P. mirabilis*, *S. aureus*, *E. coli* and *P. aeruginosa* were determined according to the CLSI broth microdilution method [21]. Briefly, serial two-fold dilutions of each WOA in MHB were performed in a 96-well plate in triplicate wells. The inoculum to be tested was prepared by dilution of actively growing overnight broth cultures in MHB to provide a final bacterial density of 5 x 10⁵ cfu mL⁻¹. Bacterial suspensions were added to each well and the plates were incubated for 24 h at 37°C. Positive growth and negative sterility controls were also included. The MIC was determined as the lowest concentration of WOA showing no visible signs of turbidity. The minimum bactericidal concentration (MBC) of each WOA was then determined by spreading 10 µL of suspension from wells that showed no visible signs of bacterial growth onto Mueller–Hinton agar (MHA) plates (low swarm agar (LSW) plates were used for *P. mirabilis*). The agar plates were incubated at 37°C for 24 h in a static incubator and then examined for 99.9% killing. MIC and MBC assays were carried out on three independent occasions [22].

Minimum biofilm eradication concentration (MBEC) values of WOAs against *P. mirabilis*, *S. aureus*, *E. coli* and *P. aeruginosa* were determined using the Calgary Biofilm Device according to the MBEC assay protocol supplied by the manufacturer [23]. Biofilms were grown over 24 h on peg lids of MBEC plates at 37°C. After rinsing for 10 seconds in PBS, the peg lids were

exposed to serial dilutions of each WOA in MHB in triplicate wells of a challenge plate. Positive growth and negative sterility controls were also included. The challenge plate was left standing for 30 min at room temperature to equilibrate before incubating for 24 h at 37°C. The pegs were then rinsed for 10 sec in PBS before placing the peg lid onto a recovery plate containing neutraliser-supplemented MHB (final concentration in each well: 0.125% w/v L-histidine, 0.125% w/v L-cysteine and 0.25% w/v reduced glutathione). After 30 min, the plates were sonicated for 30 minutes to dislodge the biofilms from the pegs. The peg lid was removed, replaced with a sterile non-pegged lid and the recovery plate was incubated at 37°C overnight. The MBEC was recorded as the lowest concentration of each WOA to visibly prevent growth in wells of the microtitre plate. MBEC assay triplicates were repeated on two independent occasions [24].

For each acid, MIC, MBC and MBEC values were expressed in terms of the concentration of total acid and the concentration of undissociated acid. The latter concentration was calculated using Equation 1, which is a rearrangement of the Henderson-Hasselbalch equation [25]:

$$[\text{Undissociated acid}] = [\text{Total acid}] / (1 + 10^{\text{pH} - \text{pK}_a}) \quad [\text{Equation 1}]$$

where:

pK_a = acid dissociation constant

pH = pH of the media at the MIC, MBC and MBEC values

Checkerboard assay

FICI values of combinations of WOAs and BKZ against the four uropathogens were determined by the checkerboard assay [9, 26, 27]. Briefly, serial two-fold dilutions of the first agent (compound A) were performed across the columns and the second agent (compound B) performed across the rows of a 96-well plate. Columns 1 - 12 of row H contained two-fold dilutions of compound A only. Rows A – H in column 12 contained two-fold dilutions of compound B only. Individual wells were inoculated with actively growing overnight broth cultures diluted with MHB to 1×10^6 cfu mL⁻¹, as verified by viable count, to provide a final inoculum density of 5×10^5 cfu mL⁻¹. The plate was incubated at 37°C overnight in an orbital incubator at 100 rpm. FICI values were calculated according to Equation 2:

$$FICI = FIC_1 + FIC_2 \quad \text{[Equation 2]}$$

where:

FIC_1 = MIC of compound A in combination / MIC of compound A alone

FIC_2 = MIC of compound B in combination / MIC of compound B alone

A FICI value <0.5 indicates synergism between the two compounds while a value >4 indicates antagonism. Between 0.5 – 4 indicates additive or no effect on the antibacterial activity [9].

Time-kill assays

Time-kill assays were performed according to CLSI methods [21]. The turbidity of bacterial cultures grown to the logarithmic phase was adjusted in MHB to obtain a final inoculum density of 5×10^5 cfu mL⁻¹, as verified by viable count. Media was supplemented with WOAs, alone and in combination, at concentrations corresponding to previously determined MBCs. Growth controls with no WOA were also included. Test and control flasks were incubated at 37°C in an orbital incubator. Viable counts and pH measurements were performed at 0, 1, 2, 3, 4, 6 and 24 h. Bactericidal activity was defined as a ≥ 3 -log₁₀ decrease in bacterial density after 24 h contact with the WOA compared to the starting inoculum and, conversely, bacteriostatic activity was defined as a ≤ 3 -log₁₀ decrease in viable bacterial density after 24 h contact [21, 22]. Time-kill assays were repeated on three independent occasions and representative curves are displayed.

Effect of WOAs on biofilm formation and eradication

Firstly, the efficacy of citric acid and propionic acid, alone and in combination, against formation of biofilms by *P. mirabilis* or *S. aureus* on silicone substrates was assessed. Silicone samples (2 cm x 2 cm) were placed vertically in wells of a 12-well plate. 3 mL aliquots of overnight broth cultures of *P. mirabilis* or *S. aureus* with density of 1×10^6 cfu mL⁻¹ in MHB were added to individual wells. Test wells contained citric acid or propionic acid, alone and in combination, at concentrations corresponding to the previously determined MBC values, whereas control wells were prepared in the absence of WOAs. The plate was incubated for 24 h in an orbital incubator at 37°C. After 24 h, the samples were rinsed twice in sterile dH₂O to remove non-adherent bacteria and then submerged in 3 mL crystal violet solution (0.5% v/v) for 15 min at room temperature to stain adherent bacteria. Samples were then rinsed three times in sterile dH₂O to remove excess crystal violet stain. After air drying, the samples were

submerged in 3 mL ethanol to release the crystal violet dye. Absorbance of the resulting solutions was measured at 595 nm with a UV spectrometer (BMG Labtech FLUOstar Omega). Two replicates were performed on three independent occasions [28, 29].

Secondly, the efficacy of citric acid and propionic acid, alone and in combination, against established biofilms of *P. mirabilis* or *S. aureus* on silicone substrates was assessed. Biofilms were grown on silicone substrates over 24 h by incubation of samples placed vertically in wells of a 12-well plate with 3 mL aliquots of overnight broth cultures of *P. mirabilis* or *S. aureus* with density of 1×10^6 cfu mL⁻¹ in MHB. Following biofilm formation, citric acid and propionic acid, alone and in combination, at concentrations corresponding to the previously determined MBC values were added to the test wells. The same volume of MHB with no WOA was added to control wells. Following a 4 h incubation period silicone samples were rinsed twice in sterile dH₂O to remove non-adherent bacteria and the adherent bacteria were then stained with crystal violet and the biofilm quantified as previously described.

Fluorescence imaging

Biofilms of *P. mirabilis* and *S. aureus* were grown as described above, treated with citric acid and propionic acid, alone and in combination, at concentrations corresponding to the previously determined MBC values for 4 h, rinsed three times in tris-buffered saline and immersed in Live/Dead BacLight™ in the dark for 30 min followed by immediate examination using a fluorescence microscope (GXM-L3201 LED, GX Optical, Suffolk, UK) with a 40x objective. ImageJ software was used for quantification of the percentage surface area covered by adherent bacteria using nine randomly selected fields of view per sample. Areas corresponding to the viable bacteria (coloured green) and the dead bacteria (coloured red) were individually calculated [30, 31]. Corresponding cells in their planktonic state were also treated with the WOAs for 4 h and stained with Live/Dead BacLight™. Stained cells (5 µL) were spotted onto a microscope slide, covered with a coverslip and the slide imaged as described above [32, 33].

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 8.0 for Mac (GraphPad Software Inc., San Diego, USA). Statistical differences in A₅₉₅ values between untreated and WOA-treated biofilms, and in live/dead ratios between the untreated and WOA-treated cells were analysed by a one-way ANOVA, whereas differences in the extent of biofilm coverage between

the untreated and WOA-treated surfaces were statistically analysed by a two-way ANOVA, with $p < 0.05$ denoting significance and $n = 9$. Where a statistically significant difference was identified, Tukey's multiple comparison post-hoc test was carried out.

Results and Discussion

MIC, MBC and MBEC values of WOAs

The antibacterial activity of WOAs (citric acid, malic acid, propionic acid, mandelic acid, lactic acid, benzoic acid, pyruvic acid and hippuric acid) against nosocomial uropathogens (*S. aureus*, *P. mirabilis*, *P. aeruginosa* and *E. coli*) was investigated through determination of MIC, MBC and MBEC values. Values are reported in Table I as the concentration of total acid and the concentration of undissociated acid.

As shown in Table I, all tested WOAs displayed bactericidal activities against *S. aureus*, *P. mirabilis*, *P. aeruginosa* and *E. coli* in their planktonic and biofilm modes of growth. As expected, higher concentrations of WOAs were required for biofilm eradication of most species due to the presence of the exopolymeric matrix, with resultant limited penetration of antibacterial agents, and the phenotypic changes, altered metabolic activity and adaptive stress responses characteristic of biofilm-associated cells [14]. While biofilm cells have previously demonstrated up to 1000-fold greater resistance to antimicrobial agents than their planktonic counterparts, the MBEC values reported herein were a maximum four-fold higher than the MBC values [34]. Furthermore, reduced susceptibility of Gram-negative bacteria to WOAs than their Gram-positive counterparts has previously been reported, due to the presence of the outer hydrophobic membrane which can block the entry of WOAs of low lipid solubility, such as malic acid [35-38]. The WOAs tested herein, however, exhibited a similarly potent action against Gram-positive and -negative bacteria.

Efficacy of WOAs has also been reported to vary according to the compound's molecular mass. Eswaranandam *et al.* (2004) previously attributed the higher antimicrobial activity of malic acid (134.09 Da) and lactic acid (90.08 Da) to the more ready permeation of these smaller molecules, in their undissociated states, through the bacterial membrane than larger molecules of, for example, citric acid (192.13 Da) [39]. Moreover, the antibacterial activity of WOAs is widely accepted to be dependent on pH, with this activity commonly assumed to result from acids in their undissociated forms [13, 15]. The triprotic acid, citric acid, has recently been demonstrated to eradicate mucoid *P. aeruginosa mucA* biofilm bacteria at pH values $< \text{pKa}1$

with no efficacy displayed at pH values > pKa2. At pH values between the pKa1 and pKa2 values, citric acid displayed similar activity to many membrane-targeting drugs, such as EDTA, and killed the metabolically inactive cells in the interior of the microcolonies with no effect on the active cells on the periphery [14]. For many of the WOAs tested herein, the concentration of undissociated acid at the MIC, MBC and MBEC values was significantly lower than the total acid concentration. For example, at the MIC of hippuric acid against *E. coli* (4.18 mg mL⁻¹) the undissociated acid concentration was 0.0004 mg mL⁻¹, and at the MBEC of benzoic acid against *S. aureus* less than 0.25% of the acid was in its undissociated state. The reported antimicrobial activity of dissociated molecules, together with studies demonstrating the absence of a correlation between acid potency and pKa, highlight the multi-mechanistic activity of WOAs, with this combination of effects, in addition to acidification of the cell interior, still to be confirmed [19, 40, 41].

Checkerboard assay

The antibacterial activity of the WOAs in combination with each other and the antiseptic BKZ was investigated by checkerboard assays and the determined FICI values against *S. aureus*, *P. mirabilis*, *P. aeruginosa* and *E. coli* are shown in Table II.

All tested combinations demonstrated synergistic or additive effects, with no antagonism. MIC values of the WOAs were reduced up to 250-fold when used in combination compared to when used individually. For example, the MIC of pyruvic acid against *P. mirabilis* was 1.38 mg mL⁻¹, however, upon the addition of lactic acid, this value decreased to 0.0055 mg mL⁻¹. All WOA combinations showed synergistic activity against *P. mirabilis*, with the lowest FICI values of 0.03, 0.04 and 0.04, indicating the most promising synergistic combinations, obtained for propionic acid/lactic acid, propionic acid/citric acid, and propionic acid/pyruvic acid mixtures, respectively. This corresponds to significant reductions in MIC values for propionic acid from 0.69 mg mL⁻¹ to 0.0215 mg mL⁻¹, lactic acid from 1.41 mg mL⁻¹ to 0.005 mg mL⁻¹, citric acid from 1.50 mg mL⁻¹ to 0.01175 mg mL⁻¹ and pyruvic acid from 1.38 mg mL⁻¹ to 0.0055 mg mL⁻¹. Similarly, all of the tested combinations showed synergistic activity against *S. aureus*, with propionic acid/lactic acid and propionic acid/citric acid combinations again demonstrating the most promising synergistic activities (FICI values of 0.03 and 0.05 respectively). In contrast, while no synergistic combinations were identified against *P. aeruginosa*, the MIC values were, in most cases, reduced ≥ 2 -fold. These findings indicate that the presence of propionic, lactic

or citric acid facilitated the actions of other WOAs and the tested antiseptic, BKZ, lowering the concentrations required to inhibit bacterial growth. Lactic acid has previously been reported to increase permeability of Gram-negative bacterial membranes, with this permeabilising activity effectively potentiating the antibacterial activity of other agents including copper and sodium lauryl sulphate [16, 42]. Citric acid has also been reported to destabilise bacterial membranes, which may therefore promote the membrane translocation of other WOAs, thus explaining the observed synergistic activity [17].

Investigated combinations of rifampicin, sparfloxacin and triclosan impregnated within silicone catheters have previously been reported to have greater efficacy in preventing bacterial growth than clinically employed antimicrobial catheters, with uropathogen colonisation *in vitro* inhibited for 7 to 12 weeks and 1 to 3 days, respectively [43]. Promising efficacy has also been reported with combinations of chlorhexidine, silver sulfadiazine and triclosan [44]. The findings of this study suggest promising potential for use of WOA combinations as urgently-needed alternatives to antibiotics for prevention of CAUTIs.

Time-kill assays

The synergistic interaction between citric acid and propionic acid was further studied by time-kill assays using concentrations representing MBC values of the agents when used alone.

As shown in Figure 1, citric acid and propionic acid were bactericidal against *E. coli* after 24 h when used individually, whereas citric acid demonstrated bactericidal activity against *P. aeruginosa* after 1 h. In contrast, these agents displayed a bacteriostatic activity against *S. aureus* and *P. mirabilis* when used alone. Combinations of citric acid and propionic acid demonstrated enhanced bactericidal activity and more rapid kill kinetics against all four pathogens than when each agent was used individually. In the presence of the citric acid/propionic acid combination, there was a $\geq 3\text{-log}_{10}$ decrease in the densities of *S. aureus*, *P. mirabilis*, *P. aeruginosa* and *E. coli* compared to the starting inoculum after 24 h, 6 h, 1 h and 6 h, respectively. Interestingly, this combination demonstrated an additive effect against *P. aeruginosa* in the checkerboard assay but synergistic activity, as defined by $\geq 2\text{-log}_{10}$ decrease in the colony count in the presence of the combination compared to the most active agent on its own, after 1 h in the time-kill assay [45]. With regards to media pH, addition of the agents decreased the pH from $\sim\text{pH } 7$ to $\sim\text{pH } 4$, with no significant differences in pH

observed between the media containing the WOA combinations compared to the media with the individual acids.

Effect of WOAs on biofilm formation and eradication

The absorbance of crystal violet solutions obtained following staining of biofilms on silicone substrates and elution of the retained stain was used to quantify biofilm biomass [46]. The efficacy of citric acid and propionic acid, alone and in combination, on prevention of biofilm formation and on eradication of established biofilms is shown in Figures 2 (a) and (b), respectively.

Significant reductions in biofilm biomass were detected after 24 h incubation of *S. aureus* and *P. mirabilis* cells with citric acid ($A_{595} = 0.118$ and 0.0758 , respectively) and propionic acid ($A_{595} = 0.152$ and 0.0923 , respectively) at concentrations corresponding to their MBC values compared with the relative controls ($A_{595} = 0.520$ and 0.408 , respectively). Furthermore, no significant differences were demonstrated between the acids alone and in combination, with respective A_{595} values of 0.122 and 0.058 for *S. aureus* and *P. mirabilis* biofilms after 24 h incubation with the WOA combination.

The acids were also tested for their ability to eradicate preformed biofilm. Both acids alone displayed killing activity against preformed biofilms of *S. aureus* and *P. mirabilis*, with significant reductions in A_{595} values from 0.521 for untreated *P. mirabilis* biofilms to 0.223 and 0.340 after 4 h treatment with citric acid and propionic acid, respectively. Moreover, the decrease in biomass was significantly greater for the acid combination than that achieved by either acid alone, suggesting synergism between the two compounds, with absorbance values for biofilms of *P. mirabilis* reduced to 0.162 .

This study demonstrates the potential of the selected WOAs to inhibit biofilm formation on silicone substrates, representative of catheter surfaces which are subject to bacterial colonisation, at their MBC values. These findings suggest the efficacy of the WOAs against biofilm formation was due to their killing activity against planktonic cells. The acids, alone and in combination, were, however, less effective against preformed biofilms highlighting the importance of inhibiting biofilm development and suggesting a key role for these agents in the

prevention of device-associated infections through, for example, localised release at the device surface [47].

Fluorescence imaging

Fluorescence microscopy images of planktonic *S. aureus* and *P. mirabilis* cells stained with Live/Dead BacLight™ following 4 h treatment with citric acid and propionic acid are shown in Figure 3 (a). Live/Dead BacLight™ contains a combination of two stains, SYTO-9, a green fluorescent nucleic acid stain which stains live cells, and propidium iodide (PI), a red fluorescent stain which only enters cells with permeabilised cytoplasmic membranes and intercalates with double stranded DNA [33, 48, 49]. The untreated control cells appeared predominantly green, indicating live cells, whereas more red fluorescence was observed with the WOA-treated cells, indicating bacterial membrane damage. The relative percentage values of live and dead cells were quantified from the fluorescence microscopy images using ImageJ software and are shown in Figure 3 (b).

A significant degree of membrane damage, and death, of both the Gram-positive and -negative pathogens in their planktonic states was induced by the WOAs, alone and in combination, with the proportion of dead cells increasing from ~20% to ≤60%. Interestingly, the live/dead ratios of both pathogens were similar following 4 h treatment with citric acid and propionic acid, therefore indicating that the acids induced a similar degree of membrane damage. Furthermore, the extent of membrane-induced damage was similar when the acids were used alone and when they were used in combination in contrast to the findings of the time-kill assay where the acid combination demonstrated enhanced bactericidal activity than either agent alone. While disruption of bacterial membranes by citric acid has previously been reported, this is the first study demonstrating a potential mechanism of action for propionic acid involving bacterial membrane damage [17]. Moreover, the extent of WOA-induced membrane damage was similar for the Gram-positive and negative pathogens despite their significantly different wall properties [37]. Similar activity against Gram-positive and -negative pathogens, mediated by membrane damage, has previously been reported for the naturally-occurring antioxidant, curcumin I [33].

In addition, the extent of biofilm coverage on silicone substrates following 4 h treatment with the WOAs was quantitatively analysed and live/dead ratios were calculated to determine the proportion of live and dead cells within the surface-attached bacterial communities, as shown in Figure 4.

No significant differences were observed in the extent of biofilm coverage between the untreated and WOA-treated surfaces, as shown in Figure 4. Similar to the planktonic cells, no significant differences in live/dead ratios were demonstrated between biofilm cells treated with either acid, and between the agents used alone and in combination. As expected, live/dead ratios of cells in the biofilm communities following WOA treatment were higher than their planktonic counterparts as a result of the increased resistance of biofilms to administered antimicrobials [34].

The bacterial biofilm communities formed on the control were observed as green microcolonies in Figure 4 in contrast to the individual green cells on the control surface in Figure 3. By staining the treated cells with PI, which cannot penetrate intact cell membranes, we show that the WOAs disrupted the integrity of the bacterial membrane. In addition, the permeabilising ability of the WOAs was much greater against cells in their planktonic states than against the biofilm communities, thereby highlighting the importance of infection prevention. Disruption of bacterial membranes, which usually function as a barrier to limit penetration of antibiotics or antimicrobial agents, has important implications for potential use of WOAs to sensitise bacterial cells to other administered agents, including those that are too large or lipophilic to penetrate intact bacterial membranes [50].

Conclusions

All WOAs tested herein displayed bactericidal activities against the uropathogens, *P. mirabilis*, *S. aureus*, *E. coli* and *P. aeruginosa*, in their planktonic and biofilm modes of growth. Moreover, MIC values were reduced up to 250-fold when the acids were used in combination. The membrane permeabilising activity of the WOAs could be exploited to increase cellular uptake, and therefore efficacy, of other antimicrobial substances. These multi-mechanistic combinations with the WOA potentiators have promising applications for the prevention and treatment of hospital-acquired infections, allowing use of lower concentrations of antimicrobial

agents and thereby preventing potential toxicity issues and the development of bacterial resistance.

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Conflict of interest

None declared.

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Tables

Table I. MIC, MBC and MBEC values for WOAs against *S. aureus*, *P. mirabilis*, *E. coli* and *P. aeruginosa*

<i>S. aureus</i> ATCC 29213							<i>P. mirabilis</i> ATCC 51286					
	MIC ^a	MIC ^b	MBC ^a	MBC ^b	MBEC ^a	MBEC ^b	MIC ^a	MIC ^b	MBC ^a	MBC ^b	MBEC ^a	MBEC ^b
	(mg mL ⁻¹)						(mg mL ⁻¹)					
Citric	0.75	0.0176	1.50	0.0109	3.00	0.746	1.50	0.139	1.50	0.139	6.00	0.956
Mandelic	2.38	0.168	2.38	0.168	2.38	0.168	2.38	0.230	2.38	0.230	4.75	3.37
Malic	1.05	0.002	2.09	0.786	4.19	2.75	2.09	0.186	2.09	0.186	8.38	5.50
Propionic	0.69	0.0577	2.75	1.49	5.50	4.06	0.69	0.0466	1.38	0.761	1.38	0.761
Lactic	1.41	0.0147	2.82	1.06	5.63	3.86	1.41	0.0005	2.82	1.06	5.63	3.83
Hippuric	2.06	0.128	4.13	0.128	4.13	1.06	2.06	0.0061	4.13	0.471	4.13	0.471
Benzoic	1.75	0.0043	1.75	0.0043	1.75	0.0043	0.88	0.0004	1.75	0.0037	3.50	1.19
Pyruvic	1.38	0.0022	1.38	0.0022	2.75	1.05	1.38	0.0002	1.38	0.0002	2.75	0.376
<i>P. aeruginosa</i> 679							<i>E. coli</i> NSM59					
	MIC ^a	MIC ^b	MBC ^a	MBC ^b	MBEC ^a	MBEC ^b	MIC ^a	MIC ^b	MBC ^a	MBC ^b	MBEC ^a	MBEC ^b
	(mg mL ⁻¹)						(mg mL ⁻¹)					
Citric	1.50	0.06	3.00	0.998	12.00	5.04	3.00	0.461	6.00	2.79	12.0	8.29
Mandelic	2.38	0.208	2.38	0.208	4.75	2.10	2.38	0.132	2.38	0.132	4.75	2.08
Malic	1.05	0.0449	4.19	0.989	8.38	4.57	2.09	0.348	4.19	2.22	8.38	6.30
Propionic	0.69	0.104	1.38	0.754	1.38	0.753	0.69	0.0308	2.75	1.47	5.50	4.04
Lactic	1.41	0.0001	2.82	0.777	5.63	3.88	2.82	0.0001	5.63	3.80	5.63	3.80
Hippuric	2.06	0.0001	4.13	0.0001	4.13	0.727	4.13	0.0004	4.13	0.0004	8.25	0.903
Benzoic	1.75	0.0005	1.75	0.0005	3.50	1.12	1.75	0.0009	1.75	0.0009	3.50	1.19
Pyruvic	1.38	0.0001	2.75	0.308	2.75	0.308	1.38	0.0001	2.75	0.369	5.50	2.34

^aConcentration of total acid; ^bConcentration of undissociated acid

Table II. FICI values for combinations of citric acid, lactic acid, propionic acid, pyruvic acid and BKZ

	Antibacterial combination	MICs alone (mg mL ⁻¹)	MICs in combination (mg mL ⁻¹)	FICI	Outcome
<i>S. aureus</i> ATCC 29213	Citric/lactic	0.75/1.41	0.188/0.005	0.25	Synergistic
	Citric/BKZ	0.75/0.002	0.01175/0.0005	0.26	Synergistic
	Lactic/BKZ	1.41/0.002	0.005/0.0005	0.24	Synergistic
	Propionic/pyruvic	0.69/1.38	0.002/0.087	0.07	Synergistic
	Propionic/BKZ	0.69/0.002	0.002/0.0005	0.24	Synergistic
	Pyruvic/BKZ	1.38/0.002	0.087/0.0005	0.30	Synergistic
	Citric/propionic	0.75/0.69	0.01175/0.0215	0.05	Synergistic
	Citric/pyruvic	0.75/1.38	0.01175/0.087	0.08	Synergistic
	Lactic/propionic	1.41/0.69	0.005/0.0215	0.03	Synergistic
	Lactic/pyruvic	1.41/1.38	0.005/0.087	0.07	Synergistic
<i>P. mirabilis</i> ATCC 51286	Citric/lactic	1.50/1.41	0.188/0.01	0.13	Synergistic
	Citric/BKZ	1.50/0.13	0.75/0.0625	1.00	Additive
	Lactic/BKZ	1.41/0.13	1.41/0.0625	1.50	Additive
	Propionic/pyruvic	0.69/1.38	0.0215/0.0055	0.04	Synergistic
	Propionic/BKZ	0.69/0.13	0.344/0.0005	0.50	Synergistic
	Pyruvic/BKZ	1.38/0.13	0.6875/0.0005	0.50	Synergistic
	Citric/propionic	1.50/0.69	0.01175/0.0215	0.04	Synergistic
	Citric/pyruvic	1.50/1.38	0.01175/0.087	0.07	Synergistic
	Lactic/propionic	1.41/0.69	0.005/0.0215	0.03	Synergistic
	Lactic/pyruvic	1.41/1.38	0.09/0.0055	0.07	Synergistic
<i>P. aeruginosa</i> 679	Citric/lactic	1.50/1.41	0.75/0.35	0.75	Additive
	Citric/BKZ	1.50/0.01	0.75/0.0015	0.65	Additive
	Lactic/BKZ	1.41/0.01	1.41/0.0015	1.15	Additive
	Propionic/pyruvic	0.69/1.38	0.172/0.69	0.75	Additive
	Propionic/BKZ	0.69/0.01	0.69/0.0015	1.15	Additive
	Pyruvic/BKZ	1.38/0.01	0.0106/0.006	0.61	Additive
	Citric/propionic	1.50/0.69	0.375/0.344	0.75	Additive
	Citric/pyruvic	1.50/1.38	0.75/0.344	0.75	Additive
	Lactic/propionic	1.41/0.69	0.703/0.344	1.00	Additive
	Lactic/pyruvic	1.41/1.38	0.703/0.344	0.75	Additive
<i>E. coli</i> NSM59	Citric/lactic	3.00/2.82	0.75/0.703	0.50	Synergistic
	Citric/BKZ	3.00/0.0025	0.1875/0.013	0.58	Additive
	Lactic/BKZ	2.82/0.0025	1.406/0.0015	0.56	Additive
	Propionic/pyruvic	0.69/1.38	0.172/0.69	0.75	Additive
	Propionic/BKZ	0.69/0.025	0.0025/0.013	0.52	Additive
	Pyruvic/BKZ	1.38/0.025	0.344/0.0015	0.31	Synergistic
	Citric/propionic	3.00/0.69	1.50/0.34	0.99	Additive

	Citric/pyruvic	3.00/1.38	1.50/0.344	0.75	Additive
	Lactic/propionic	2.82/0.69	1.406/0.0859	0.62	Additive
	Lactic/pyruvic	2.82/1.38	1.406/0.69	1.00	Additive

Figures

a, ii

b, i

b, ii

Figure legends

Figure 1. Kill kinetics of citric acid and propionic acid, alone and in combination. Concentrations of citric acid: 1.50 mg mL⁻¹, 1.50 mg mL⁻¹, 3.00 mg mL⁻¹, 6.00 mg mL⁻¹, and propionic acid: 2.75 mg mL⁻¹, 1.38 mg mL⁻¹, 1.38 mg mL⁻¹, 2.75 mg mL⁻¹ were used against (a) *S. aureus*, (b) *P. mirabilis*, (c) *P. aeruginosa* and (d) *E. coli* respectively. WOAs were added at t=0. pH of the media was measured at every time point and values are displayed by the symbols with no connecting lines. Controls with no WOAs were included in every assay. Each time-kill assay was repeated on three independent occasions and representative curves are displayed.

Figure 2. Efficacy of WOAs (alone and in combination) on (a) biofilm formation and (b) preformed biofilms of (i) *S. aureus* and (ii) *P. mirabilis*. Error bars represent S.D.'s of mean values (n = 6).

Figure 3. (a) Fluorescence microscopy images and (b) relative percentage of live and dead cells following 4 h treatment of planktonic (i) *S. aureus* and (ii) *P. mirabilis* with citric acid and propionic acid. Bacteria were stained with Live/Dead BacLight™. Scale bar = 50 µm.

Figure 4. (a) Fluorescence microscopy images and (b) biofilm coverage on the silicone substrates indicating the proportions of live and dead cells following 4 h treatment of biofilms of (i) *S. aureus* and (ii) *P. mirabilis* with citric acid and propionic acid. Bacteria were stained with Live/Dead BacLight™. Scale bar = 50 µm.