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Inactivation of the Bacterial Pathogens *Staphylococcus pseudintermedius* and *Acinetobacter baumannii* by Butanoic Acid

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Abstract

Aims

This study was performed to evaluate the efficacy of butanoic acid against bacterial pathogens including *Acinetobacter baumannii* and *Staphylococcus pseudintermedius*.

Methods and Results

Vegetative bacteria were exposed to butanoic acid *in vitro* and log reduction was quantified using viable count assays. The maximum (8 and 9) log inactivation was determined by qualitatively assaying for growth/no-growth after a 48-h incubation (37°C). Membrane integrity after exposure to butanoic acid was determined by propidium iodide staining, scanning electron microscopy, membrane depolarization and inductively coupled plasma analysis. Cytosolic pH was measured by 5-(6-)carboxyfluorescein succinimidyl ester.

Conclusions

Inhibitory concentrations of butanoic acid ranged between 11 and 21 mmol l^{-1} for Gram-positive and Gram-negative species tested. The maximum log reduction of *A. baumannii* was achieved with a 10-s exposure of 0·50 mol l^{-1} of butanoic acid. *Staphylococcus pseudintermedius* required 0·40 mol l^{-1} of butanoic acid to achieve the same level of reduction in the same time period. Inactivation was associated with membrane permeability and acidification of the cytosol.

Significance and Impact of the Study

Antibiotic resistance among bacterial pathogens necessitates the utilization of novel therapeutics for disinfection and biological control. These results may facilitate the development of butanoic acid as an effective agent against a broad-spectrum of antibiotic-resistant bacterial pathogens.

Introduction

The emergence of antibiotic resistance among bacterial pathogens continues to be a major public health concern. Multidrug-resistant *Acinetobacter baumannii*, for example, is categorized within the

serious hazard level by the Centers for Disease Control and is of significant risk to public health (Dijkshoorn *et al*. **2007**). *Acinetobacter baumannii* is of particular concern to military personnel due to its prevalence during Operation Iraqi freedom, where it earned the nickname 'Iraqibacter' (Yun and Murray **2016**). *Staphylococcus pseudintermedius* is a commensal, opportunistic pathogen of companion animals such as *Canis lupus familiaris*. *Staphylococcus pseudintermedius* has acquired resistance mechanisms from methicillin-resistant *Staphylococcus aureus* (Weese and van Duijkeren **2010**) and veterinarians now consider infection by methicillin-resistant *S. pseudintermedius* (MRSP) as untreatable in dogs and cats (Weese *et al*. **2013**). Compounding this development, transfer of MRSP between companion animals and their owners has already been documented (Couto *et al*. **2016**; Pomba *et al*. **2017**). The need to develop alternative antimicrobial therapies against *S. pseudintermedius* is therefore important amongst clinicians and veterinarians alike.

Combatting the emergence of resistant strains of clinically relevant bacteria relies on a multipronged approach, one of which is the development of novel antimicrobials. Fatty acids are ubiquitous organic molecules critical to a wide array of biological processes including metabolism (Jenkins *et al*. **2015**), membrane structure (Shaikh *et al*. **2015**) and cell signalling (Kenny *et al*. **2009**). These weak organic acids also function as broad spectrum antimicrobial agents (reviewed in Desbois and Smith **2010**) and are widely utilized in natural host defence mechanisms in the intestine (Sun and O'Riordan **2013**) and on the surface of skin (Wille and Kydonieus **2003**). This antimicrobial activity is a function of two important variables: fatty acid structure and protonation state of the carboxyl group. In general, *cis*-oriented monounsaturated fatty acids with a chain length between 10 and 16 carbons appear to be most potent antimicrobials (Galbraith *et al*. **1971**; Kabara *et al*. **1972**; Feldlaufer *et al*. **1993**). However, this is likely an overgeneralization as comparisons between studies are difficult due to contrasting methodological approaches, limited solubility of longer chain acids and the variety of species tested. Importantly, antimicrobial activity requires protonation of the carboxyl group (Butkus *et al*. **2011**). For example, inactivation of *Ascaris* eggs required a pH less than the pK_a for each fatty acid tested (Butkus *et al*. **2011**). This ease of neutralization, combined with its proclivity for biodegradation (Sheridan *et al*. **2003**), provides a major advantage for the use of butanoic acid as a 'green chemistry' disinfectant (Anastas and Eghbali **2010**). These characteristics support the sustainable principles for chemical synthesis and degradation adopted by the American Chemical Society and provides a more environmentally conscious alternative to traditional, broad-spectrum disinfectants such as bleach and chlorine.

While the antimicrobial activity of fatty acids is well known, their applicability against bacterial pathogens significant to human and animal health remains largely uncharacterized. The antibacterial activity of butanoic acid was examined specifically, because it is an FDA-approved food and drug additive and classified as generally recognized as safe (**FDA 21 CFR 582.60**). It was hypothesized that treatment of pathogens with butanoic acid would result in significant inactivation via membrane disruption and acidification of the cytosol. This study aims to determine the bacteriostatic and bactericidal activity of several short-chain fatty acid (SCFA) against a variety of microbes, with focus on butanoic acid treatment of *A. baumannii* and *S. pseudintermedius*.

Materials and methods

Culture conditions and toxicity experiments

Escherichia coli (ATCC 15597), *S. pseudintermedius* (ATCC 49051) and *A. baumannii* (AB 5075 WT) were grown in nutrient broth (37°C; 125 rev min^{-1}) to a constant optical density (OD) (1·5–2·0; 600 nm) for each organism. Harvested cells were concentrated by centrifugation and resuspended in 0·1× the original volume. Acid toxicity experiments were carried out by adding 0·1 ml of cells to 0·9 ml of SCFA or HCl-amended water at pH 4 at 37°C. After exposure, cells were enumerated by dilution and the drop plate technique (Naghili *et al*. **2013**). Eight drops were counted for each dilution tube plated. Assays of 1 min or less were static, whereas those >1 min were shaken (125 rev min^{-1}). These experiments had a detection limit of ~ 4-log because inactivation activity of SCFAs cannot be neutralized during experimentation. Therefore, dilution of the reaction mixture 100X, to a working concentration below the minimum inhibitory concentration (MIC), was required to halt the inactivation activity of butanoic acid. To determine 8- and 9-log inactivation, bacteria were treated *in vitro* as above and 1 ml of cell suspension was inoculated into large volumes of nutrient broth, ensuring a working concentration of SCFA below the determined MIC. Cultures were incubated (37°C, 125 rev min^{-1}) and visually assessed for growth/no growth.

Minimum inhibitory concentrations

Minimum inhibitory concentrations of SCFAs were determined by a modification as described (Carson and Riley **1995**). Mueller–Hinton broth inoculated with bacteria was incubated (37°C, 125 rev min^{-1}) overnight. The absorbance of the culture was adjusted to 0·5 McFarland standard and further diluted such that the final absorbance yielded 1×10^5 cells per ml. The culture was used to inoculate (10 μl) a series of 1 : 2 dilutions of each SCFA in 96-well, flat-bottom plates (final volume of 160 μl). The plates were incubated at 37°C for 24 h while shaking at 125 rev min^{-1}. The OD was measured at 490 nm on a BioRad plate reader (Hercules, CA).

SEM

Cells were exposed to butanoic acid as described above, filtered (0.22 μm), fixed in 3% glutaraldehyde (0·1 mol l^{-1} phosphate buffer, pH 7·0), washed with buffer, dehydrated with ethanol sequentially (25–100%), air-dried, mounted on aluminium stubs and coated with 100–120 angstroms of gold/palladium (Denton Vacuum Desk IV sputter coater, 45 mAmp, 50 mTorr, 15 s exposure) (Bozzola **2001**). Images were obtained on a Hitachi TM3000 Tabletop Scanning Electron Microscopy (Hitachi High-Technologies Corporation, Tokyo, Japan) and image analysis was performed with Image J (Schneider *et al*. **2012**).

Membrane permeabilization

After harvest and concentration, cells were washed three times with 1 mmol l^{-1} of phosphate buffer and exposed to butanoic acid as described above, followed by two phosphate buffer washes and resuspension in 1·0 ml buffer. Propidium iodide was added to cells as described (Boswell *et al*. **1998**). The reaction vessels were statically incubated at room temperature in the dark for 10 min. Cells were pelleted and resuspended in 1·0 ml of phosphate buffer to reduce background fluorescence. Aliquots (90 μl) were pipetted in a 96-well plate and fluorescence was measured on a BioTek Synergy 4 plate reader using gen 5 1.10 software (495 nm excitation wavelength; 615 nm emission wavelength; BioTek Instruments, Winooski, VT).

Membrane leakage

Washed cells (200 μl) were added to 180 μl of acid or water. At times post-inoculation, the reaction mixture was centrifuged (14 000 ***g***) for 20 s and filtered (0·45 μm). All samples were run in duplicate and combined. The combined filtered supernatants were diluted with deionized water to a final volume of 45 ml. Control samples of each species were treated as described above by replacing butanoic acid with HCl-amended water, pH 4. The samples were digested in a microwave digester with 4 ml of ultrapure concentrated HNO_3, concentrated HCl, and 1 ml of 30% H_2O_2 using the EPA method 3015 for a final volume of 50 ml. Calibration blanks and standards were treated similarly. The samples were analyzed with inductively coupled plasma-optical emission spectrometry (ICP-OES; Optima 2100DV, PerkinElmer, Shelton, CT) for the following elements: Ca, Fe, Mg and P. The Perkin Elmer multi-element calibration standard 3 was used for Ca, Mg and Fe. Continued calibration verification was conducted with a midrange Perkin Elmer Std 3 of 0·5 mg l^{-1}. The estimated detection limits for ICP-OES were 0·100 mg l^{-1} for Ca, 0·050 mg l^{-1} for Mg and 0·010 mg l^{-1} for Fe.

Membrane depolarization

The depolarization assay was conducted by adding 25 μl of a 250-μmol l^{-1} stock solution of bis-(1,3-dibutylbarbituric acid) trimethine oxonol (bis-oxonol) to 20 ml of 1-mmol l^{-1} phosphate buffer (pH 7·0). Cells were grown and washed as described above. A 200-μl aliquot of cells was added to the buffer dye mixture, vortexed and allowed to incubate statically for 15 min. The reaction vessel was centrifuged (5 min, 14 000 ***g***) and the pellet washed twice in 0·75 ml of the dye buffer mixture. The pellet was resuspended in 1·5 ml of the dye buffer mixture and the fluorescence measured as described above with an excitation and emission wavelengths of 490 and 525 nm, respectively. After incubation, 200 μl of the reaction mixtures were added to a 96-well plate as described by Louzao *et al*. (**2004**) and monitored for 10 min. The fluorescence at a single time point was recorded after stabilization. Heat-killed controls (100°C, 10 min) were used as the positive control (Coronel-León *et al*. **2016**).

Internal pH

Cytoplasmic pH was determined as described by Breeuwer *et al*. (**1996**) and modified by Budde and Jakobsen (**2000**) and Cheng *et al*. (**2015**). Briefly, cells were grown and harvested as above, diluted (OD_{600} = 0·6), washed with 10 mmol l^{-1} of phosphate buffer and stained with 10 μmol l^{-1} of 5-(6-)carboxyfluorescein succinimidyl ester (cFSE) for 30 min (37°C). Cells were then resuspended in 0·01 mol l^{-1} of glucose for 30 min (37°C), washed in phosphate buffer three times and stored at 4°C in the dark. Preloaded cells were then washed with phosphate buffer and used to inoculate (10 μl) a series of 1 : 2 butanoic acid dilutions in a 96-well, flat bottom plate (final volume 100 μl). The fluorescent ratio (495 nm/440 nm excitation wavelength; 525 nm emission wavelength) was measured using a BioTek Synergy 4 plate reader using gen 5 1.10 software. The standard curve was generated by treating cFSE-loaded cells with 63% (v/v) ethanol to permeabilize the cells. These cells were then inoculated (10 μl) into buffer solutions ranging from pH 2 to pH 9, in 1·0 unit increments. The calibration curve was plotted by fitting the ratio 495/440 *vs* pH to a sigmoidal function (Breeuwer *et al*. **1996**). A unique calibration curve was generated at each time point measured.

Results

Antibacterial activity of SCFAs

The MIC of four SCFAs were determined for variety of bacterial species including *A. baumannii, S. pseudintermedius* and *Bacillus anthracis* Sterne (Table **1**). Inhibitory concentrations among these species range between 12 and 25 mmol l^{-1}. *Bacillus subtilis* and *Bacillus megaterium* vegetative cells show particular susceptibility to hexanoic acid with MICs of 0·95 and 2·4 mmol l^{-1} respectively. *Staphylococcus pseudintermedius* and *B. subtilis* were most susceptible to butanoic acid, with 11 mmol l^{-1} sufficient for growth inhibition (Table **1**). Growth was observed when bacteria were treated with HCl-amended water, pH 4 as a control (data not shown).

Table 1. Minimum inhibitory concentrations of short chain fatty acids (mmol l^{-1}) at 37°C, pH 4·0, 24 h

Organism	Gram stain	Butanoic	Hexanoic	Pentanoic	Propanoic
Acinetobacter baumannii a	−	21	19	25	23
Bacillus anthracis Sterne strain	+	21	12	12	23
Bacillus subtilis	+	11	0·95	12	12
Bacillus megaterium	+	21	2·4	12	23
Escherichia coli	−	21	ND	13	23
Staphylococcus aureus	+	21	19	25	23
Staphylococcus epidermidis	+	21	19	25	23
Staphylococcus intermedius	+	21	19	25	23
Staphylococcus pseudintermedius	+	11	19	25	23

ND, not determined.

[a] AB 5075 WT.

Bactericidal activity of butanoic acid was determined against a variety of Gram-positive and Gram-negative bacterial species including *A. baumannii* and *S. pseudintermedius* (Table **2**). Bacterial cells (~10^9) were exposed to various concentrations of butanoic acid and then inoculated into large volumes of NB media in order to halt any bacteriostatic activity. After a 48-h incubation at 37°C, cultures were qualitatively assayed for growth. As summarized in Table **2**, A*. baumannii* exposed to 0·50 mol l^{-1} of butanoic acid for 10 s resulted in an 8-log inactivation, demonstrating inactivation of the entire 10^8 CFU sample. *Staphylococcus pseudintermedius* was noticeably more susceptible to butanoic acid, requiring a 10-s exposure to 0·40 mol l^{-1} to achieve a 9-log inactivation. However, this increased susceptibility was not broadly applicable to Gram-positive species as *Micrococcus luteus* treated with 0·70 mol l^{-1} failed to inactivate the entire sample of 10^9 CFU (Table **2**). In all cases, complete inactivation of the sample was dose-dependent (Table **2**). These data demonstrated that butanoic acid is an effective bactericidal agent capable of inactivating both Gram-positive and Gram-negative bacteria *in vitro*.

Table 2. Time required for complete inactivation (9-log) of log-phase vegetative cells with butanoic acid (37°C) *in vitro*

Butanoic acid (mol l^{-1})	*Acinetobacter baumannii* [a]	*Escherichia coli*	*Staphylococcus pseudintermedius*	*Enterococcus saccharolyticus*	*Micrococcus luteus*

0·70	ND	ND	ND	ND	>10 s[b]
0·60	ND	10 s	ND	10 s	>40 s
0·50	10 s	15 s	ND	30 s	>90 s
0·40	6 min	4 min	10 s	>5 min	>5 min
0·30	30 min	10 min	1 min	>10 min	>30 min
0·20	2·5 h	1·5 h	2 min	>1 h	>60 min
0·10	4 h	4·25 h	40 min	ND	ND

ND, not determined.

[a] Maximum log inactivation for *A. baumannii* was eight due to a sample density limit of 10^8 CFU per ml.

[b] A 10-s exposure of *M. luteus* to 0·70 mol l^{-1} of butanoic acid was insufficient to obtain a 9-log inactivation.

The viability of *A. baumannii*, *E. coli* and *S. pseudintermedius* treated with butanoic acid was quantified over time using viable plate count methods. Log-inactivation values were determined by comparison of acid-treated cells to bacteria treated with HCl-amended water, pH 4. Between 10 and 30 s, the log inactivation of *A. baumannii* exposed to 0·25 mol l^{-1} butanoic acid increased from ~0·75 to 3·8 (Fig. **1**). The inactivation of *E. coli* increased similarly, reaching 2·2-log inactivation after 30 s with 0·30 mol l^{-1} of butanoic acid. *Staphylococcus pseudintermedius* was the most susceptible to butanoic acid exposure as treatment with 0·20 mol l^{-1} of acid for 30 s resulted in ~4-log inactivation (Fig. **1**). Increased butanoic acid concentration and exposure times were tested, resulting in >4-log reduction (data not shown). In summary, inactivation of vegetative bacteria by butanoic acid increased linearly over a 30-s exposure to butanoic acid once a concentration threshold was met.

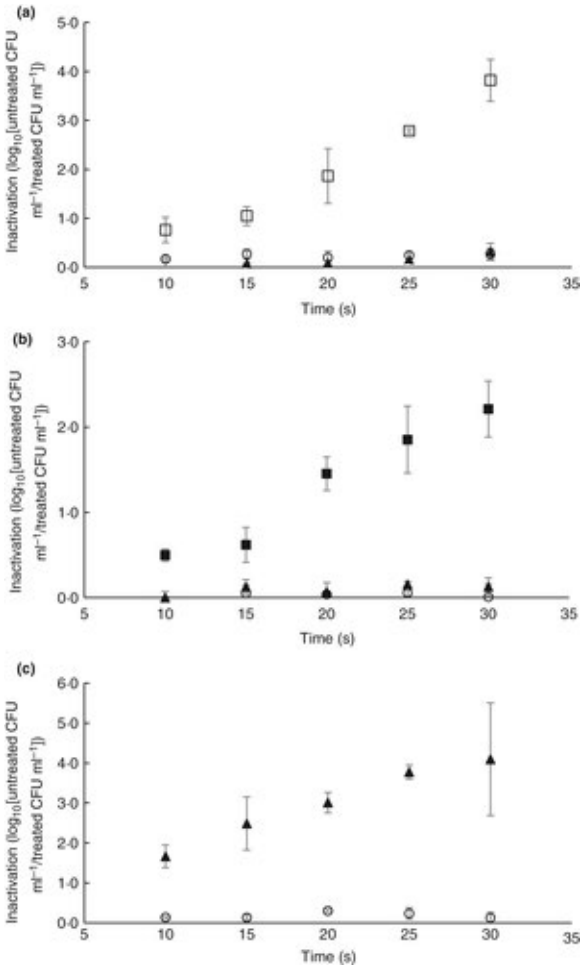

Figure 1 Inactivation curves of (a) *Acinetobacter baumannii*, (b) *Escherichia coli* and (c) *Staphylococcus pseudintermedius* with butanoic acid at 37°C. Butanoic acid concentrations: (○) 0·10 mol l^{-1}, (▲) 0·20 mol l^{-1}, (□) 0·25 mol l^{-1} and (■) 0·30 mol l^{-1}. Each point represents average inactivation (log_{10} (untreated CFU per ml/treated CFU per ml)) of three independent experiments, performed in triplicate. Error bars denote standard deviation.

Membrane integrity

Visual inspection of cell morphology was determined by scanning electron microscopy after treatment with butanoic acid. *Acinetobacter baumannii*, *E. coli* and *S. pseudintermedius* exhibited no gross morphological change after treatment with 1·2 mol l^{-1} of butanoic acid when compared to treatment with HCl-amended water, pH 4 (Fig. **2**). In accordance with this observation, OD measurements of bacterial suspensions exposed to butanoic acid for 15 min demonstrated a lack of cell lysis over this time frame (Fig. **3**). However, propidium iodide analysis clearly demonstrated that butanoic acid treatment elicited permeabilization of the plasma membrane (Fig. **4**). Time course measurements of propidium iodide fluorescence showed an increase in membrane disruption upon exposure to 0·10 mol l^{-1} of butanoic acid. Visual inspection of propidium iodide-treated bacteria confirmed disruption of the cell envelope upon exposure to 1·2 mol l^{-1} of butanoic acid (data not shown). Together, these data demonstrated that butanoic acid induced membrane damage.

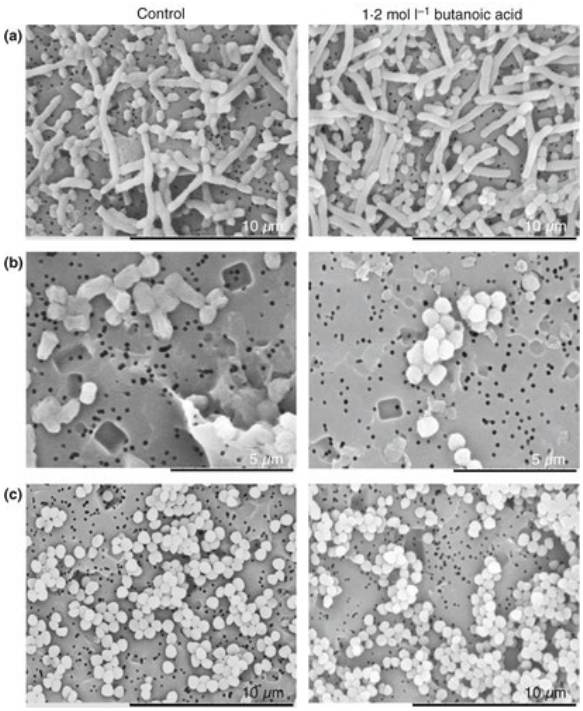

Figure 2 Scanning electron micrograph of HCl-amended water, pH 4 (control) and 1·2 mol l^{-1} of butanoic acid-treated (a) *Acinetobacter baumannii*, (b) *Escherichia coli* and (c) *Staphylococcus pseudintermedius*. Images acquired after a 60-s exposure at 37°C. Fields representative of three independent experiments.

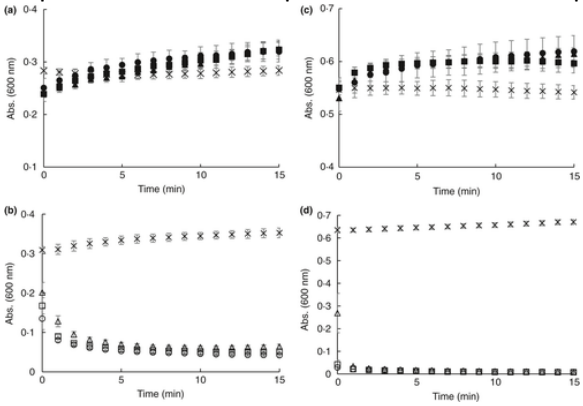

Figure 3 Absorbance (600 nm) of (a, b) *Acinetobacter baumannii* and (c, d) *Staphylococcus pseudintermedius* after treatment with (●) 340 mmol l^{-1}, (■) 170 mmol l^{-1}, and (▲) 85 mmol l^{-1} butanoic acid. Lysis controls: (○) 3% bleach, (□) 1·5% bleach, (▵) 0·75% bleach and (×) HCl-amended water, pH 4. Data points represent average of eight wells, graphs representative of three independent experiments. Error bars denote standard deviation.

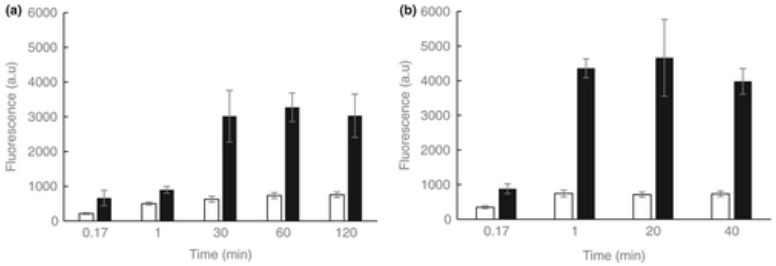

Figure 4 Fluorescence (a.u) of (a) *Acinetobacter baumannii* and (b) *Staphylococcus pseudintermedius* stained with propidium iodide (5 μg ml^{-1}). Stained bacteria were treated with (□) HCl-amended water, pH 4 and (■) 0·10 mol l^{-1} of butanoic acid for the indicated time. Data points represent average of eight wells, graphs representative of three independent experiments. Error bars denote standard deviation.

Due to the apparent permeabilization of the plasma membrane, it was hypothesized that butanoic acid treatment would result in leakage of intracellular components. To test this, *A. baumannii*, *E. coli* and *S. pseudintermedius* were exposed to butanoic acid and ICP-OEM was performed. *Acinetobacter baumannii* experienced a near complete loss of calcium and magnesium after a 2-min exposure to 0·40 mol l^{-1} of butanoic acid. The maximum leakage of iron occurred after a 6-min exposure to 0·40 mol l^{-1} of butanoic acid. *Escherichia coli* experienced a complete loss of magnesium after a 4 min exposure to 0·40 mol l^{-1} of butanoic acid. *Staphylococcus pseudintermedius* demonstrated the maximum leakage of magnesium; however, iron was unaffected by acid treatment (Table **3**). In summary, these data demonstrate that butanoic acid causes near maximum leakage of cellular components.

Table 3. Leakage of vital ions from cells exposed to butanoic acid (37°C, pH 4·0)

Organism	**Butanoic acid (mol l^{-1})**	**Time (min)**	**Ion (mg l^{-1})**		
			Ca	**Mg**	**Fe**
Acinetobacter baumannii	H_2O	6	BDL	BDL	BDL
	0·40	2	0·127	0·267	BDL
	0·40	6	0·124	0·269	0·014
	Whole cell control	NA	0·137	0·342	0·012
Escherichia coli	H_2O	6	BDL	BDL	BDL
	0·40	4	0·188	0·147	BDL
	Whole cell control	NA	BDL	0·140	BDL
Staphylococcus pseudintermedius	H_2O	2	BDL	BDL	BDL
	0·40	2	BDL	0·248	BDL
	Whole cell control	NA	BDL	0·286	0·016
H_2O			BDL	BDL	BDL
1 mmol l^{-1} PO_4 buffer			BDL	BDL	BDL

BDL, below detection limits; NA, not applicable.
Detection limits: Ca <0·100 mg l^{-1}; Mg <0·050 mg l^{-1}; Fe <0·010 mg l^{-1}.

Membrane depolarization

In addition to causing cell membrane disruption and leakage of vital materials, the presence of butanoic acid led to depolarization of the cell membrane. *Acinetobacter baumannii* and *S. pseudintermedius* were exposed to bis-(1,3-dibutylbarbituric acid)-trimethine oxonol fluorescent dye to measure change in membrane potential as reported (Breeuwer and Abee **2000**). Exposure of *A. baumannii* to lethal concentrations of butanoic acid demonstrated significant depolarization (Fig. **5**a). This activity was dose-dependent and was not observed when exposed to butanoic acid at subinhibitory concentrations. *Staphylococcus pseudintermedius* demonstrated a similar dose-dependent depolarization activity when exposed to butanoic acid (Fig. **5**b). This pattern was generally consistent with those observed for *E. coli* and *M. luteus* as well (data not shown). Together this demonstrated that butanoic acid disrupted the membrane potential of Gram-positive and Gram-negative bacterial species.

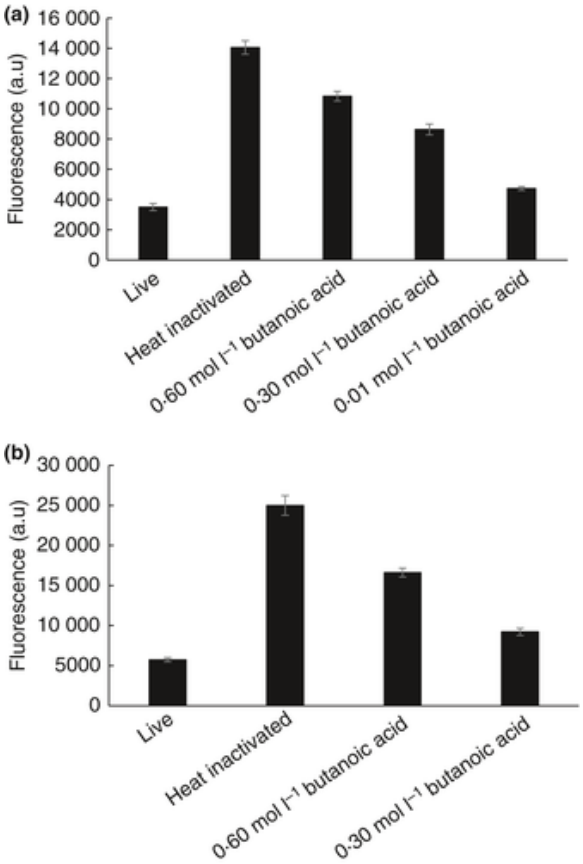

Figure 5 Membrane depolarization of (a) *Acinetobacter baumannii* and (b) *Staphylococcus pseudintermedius* measured using bis-(1,3-dibutylbarbituric acid) trimethine oxonol. Data points represent average fluorescence of six wells. Error bars denote standard deviation. Graphs representative of three independent experiments.

Internal pH

The internal pH of Gram-positive and Gram-negative species exposed to butanoic acid was measured by staining cells with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (cFSE). Prestained cells were treated with butanoic acid and the cytoplasmic pH was monitored in real time (Cheng *et al*. **2015**). *Acinetobacter baumannii* treated with sublethal concentrations of butanoic acid experienced a dose-dependent, rapid decline in cytosolic pH within 1 min of exposure (Fig. **6**a; *t* = 1). The cytosolic pH of *A. baumannii* remained relatively constant over monitoring period of 65 min monitoring period (Fig **6**a; *t* = 1 *vs t* = 65). *Staphylococcus pseudintermedius* exposure to butanoic acid resulted in an initial rapid decrease in cytosolic pH (*t* = 1) with higher concentrations of butanoic acid resulting in a greater decrease in cytosolic pH (Fig. **6**b). In contrast to *A. baumannii*, the cytoplasmic pH of *S. pseudintermedius* experienced a gradual decrease in pH over time at all acid concentrations tested (Fig. **6**b). Furthermore, this internal acidification was specific to the protonated form of the butanoic acid as HCl-amended water, pH 4 failed to cause internal acidification of *S. pseudintermedius* (Fig. **6**b) and only a mild decrease (to ~6·8) in *A. baumannii* (Fig. **6**a). *Escherichia coli* demonstrated an acidification pattern similar to *A. baumannii* (data not shown). In summary, these data demonstrate that butanoic acid causes acidification of the cytosol, even at subinhibitory concentrations in both *A. baumannii* and *S. pseudintermedius*.

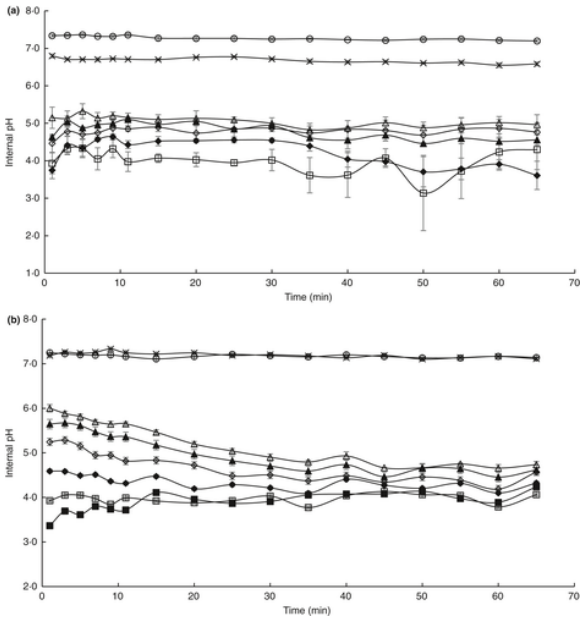

Figure 6 Internal pH of (a) *Acinetobacter baumannii* and (b) *Staphylococcus pseudintermedius* exposed to butanoic acid over time. Bacteria were exposed to (■) 100 mmol l^{-1}, (□) 50 mmol l^{-1}, (◆) 25 mmol l^{-1}, (◊) 12·5 mmol l^{-1}, (▲) 6·25 mmol l^{-1} and (▵) 3·1 mmol l^{-1} butanoic acid. Bacteria exposed to (×) HCl-amended water, pH 4 and (○) phosphate buffer pH 7 were used as controls. Data points represent average of three wells, graphs representative of three independent experiments. Error bars denote standard deviation.

Discussion

It is clear that antibiotic resistance among bacterial pathogens is a major concern to public health systems worldwide. This necessitates the need for alternative, broad-spectrum antimicrobials for human and animal health. Fatty acids are commonly used by the food industry to inhibit bacterial growth (Ricke **2003**). However, their use as disinfectants against pathogens relevant to human and animal health is not well understood. The potential use of SCFAs, such as butanoic acid, are an attractive alternative to combat emerging multidrug resistance threats such as *A. baumannii*, *E. coli* and *S. pseudintermedius*. These findings could inform the application of butanoic acid as a 'green chemistry' disinfectant to decontaminate sanitary equipment and support treatment of cutaneous infections with multidrug resistant bacteria.

These results clearly demonstrated that *A. baumannii*, *E. coli* and *S. pseudintermedius* were inactivated by butanoic acid *in vitro* (Fig. **1**). While the MIC for butanoic acid was relatively constant (between 11 and 21 mmol l^{-1}) for a variety of Gram-positive and Gram-negative bacteria, the concentration required to produce an 8- or 9-log inactivation was species dependent (Table **2**). *Staphylococcus pseudintermedius* was noticeably more susceptible to butanoic acid than other species tested; however, this does not appear to be a trend with Gram-positive species in general. For example, a 10-s exposure to 1·0 mol l^{-1} of butanoic acid failed to achieve a 9-log reduction in *M. luteus* (Table **2**). Butanoic acid produced a nonlinear dose response among all species tested – a pattern similar to many other antimicrobials (Berenbaum **1978**). As the concentration of butanoic acid decreased, the contact time required to achieve an 8- or 9-log reduction of bacteria cells increased dramatically (Table **2**). Acidic environments are known to alter the surface charge distribution causing a nonuniform distribution of cells *in vitro* (Sharma *et al*. **1985**; Dickson and Koohmaraie **1989**; Ukuku and Fett **2002**).

This phenomenon, together with the observation that butanoic acid induced clumping of bacteria (data not shown), suggested that this nonlinear response was the result of uneven exposure of individual cells to the disinfecting chemical. This would imply that pretreatment with surfactants may enhance butanoic acid-induced inactivation.

Microscopic analysis of cell morphology indicated that butanoic acid failed to elicit cellular lysis (Fig. **2**) at concentrations two to five times greater than the bactericidal concentration. SEM analysis of the cellular morphology of all species tested showed no change in morphology, demonstrating that the cell wall remained generally intact (Fig. **3**). However, membrane damage has previously been reported in bacteria after exposure to organic acid. Tuttle *et al*. (**1977**) observed membrane blebbing after exposing *Thiobacillus ferroodoxans* to butanoic acid. Sannasiddappa *et al*. (**2017**) also observed a similar morphological change after exposure of *S. aureus* to bile salts. These analyses were determined at a higher magnification and thus the possibility that butanoic acid induces a similar response in *A. baumannii* and *S. pseudintermedius* cannot be discounted.

A long-standing hypothesis for SCFA inactivation of microbes supposes that the acid passes through the plasma membrane dissociates in the more alkaline environment and acidifies the cytosol (Wojtczak and Wieckowski **1999**). This hypothesis was tested by measuring fluorescence of a pH sensitive probe, cFSE, during exposure to butanoic acid in real time. These data demonstrated that rapid acidification occurred (Fig. **4**; *t* = 2 min) followed by a steady decline in pH to ~ pH 4 (Fig. **4**; *t* = 65 min) for *S. pseudintermedius*. Importantly, this internal acidification is not simply a result of the acidic environment. *Staphylococcus pseudintermedius* exposed to HCl-amended water, pH 4 was able to maintain a neutral cytoplasmic environment, indistinguishable from an acid-free environment of phosphate buffer (pH 7). These findings support the internal acidification hypothesis; however, these data show that this internal acidification occurred at sub-inhibitory concentrations of butanoic acid. Evidence of uncoupling of cytosolic pH with inactivation and inhibitory activity has not been previously reported in the literature. As with *S. pseudintermedius*, rapid acidification of the *A. baumannii* cytoplasm was dose-dependent. However, the concentration of hydronium ions did not accumulate in the *A. baumannii* cytoplasm similarly to *S. pseudintermedius*. Both species demonstrated that cytosolic acidification is specific to the organic acid, as a neutral cytoplasm was maintained when both species were exposed to HCl-amended water, pH 4. These data suggest that internal pH may not be a complete explanation for the mechanism of inactivation.

Butanoic acid appears to be an effective SCFA capable of inhibiting growth of Gram-negative and Gram-positive pathogens including *E. coli, A. baumannii* and *S. pseudintermedius*. At sufficient species-dependent concentrations, butanoic acid inactivated as many as 10^9 bacterial cells *in vitro*. However, it is important to note that these analyses were performed on bacteria in log-phase, and in turn not representative of the growth phase common to bacteria found naturally in the environment. Future work will need to address the effectiveness of butanoic acid against pathogens in stationary phase, biofilms and part of diverse microbial communities as these conditions may alter disinfection parameters. As a broad-spectrum antimicrobial compound, butanoic acid may prove to be appropriate for treatment and protection against bacterial pathogens relevant to human health.

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Conflict of Interest

None.

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