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Published version

CAPPER-PARKIN, K.L., NICHOL, T., SMITH, T.J., LACEY, M.M. and FORBES, S. (2023). Antimicrobial and cytotoxic synergism of biocides and quorum-sensing inhibitors against uropathogenic Escherichia coli. The Journal of hospital infection, 134, 138-146.

Journal of Hospital Infection 134 (2023) 138–146

Available online at www.sciencedirect.com

Journal of Hospital Infection

journal homepage: www.elsevier.com/locate/jhin

Antimicrobial and cytotoxic synergism of biocides and quorum-sensing inhibitors against uropathogenic *Escherichia coli*

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ARTICLE INFO

Article history:
Received 29 November 2022
Accepted 11 February 2023
Available online 18 February 2023

Keywords:
Biocides
Quorum-sensing inhibitors
Uropathogenic *Escherichia coli*
Combinatorial therapies
Urinary tract infections
Catheter-associated urinary tract infections

SUMMARY

Background: Uropathogenic *Escherichia coli* (UPEC) are a primary cause of catheter-associated urinary tract infections (CAUTIs), often forming mature recalcitrant biofilms on the catheter surface. Anti-infective catheter coatings containing single biocides have been developed but display limited antimicrobial activity due to the selection of biocide-resistant bacterial populations. Furthermore, biocides often display cytotoxicity at concentrations required to eradicate biofilms, limiting their antiseptic potential. Quorum-sensing inhibitors (QSIs) provide a novel anti-infective approach to disrupt biofilm formation on the catheter surface and help prevent CAUTIs.

Aim: To evaluate the combinatorial impact of biocides and QSIs at bacteriostatic, bactericidal and biofilm eradication concentrations in parallel to assessing cytotoxicity in a bladder smooth muscle (BSM) cell line.

Methods: Checkerboard assays were performed to determine fractional inhibitory, bactericidal, and biofilm eradication concentrations of test combinations in UPEC and combined cytotoxic effects in BSM cells.

Findings: Synergistic antimicrobial activity was observed between polyhexamethylene biguanide, benzalkonium chloride or silver nitrate in combination with either cinnamaldehyde or furanone-C30 against UPEC biofilms. However, furanone-C30 was cytotoxic at concentrations below those required even for bacteriostatic activity. A dose-dependent cytotoxicity profile was observed for cinnamaldehyde when in combination with BAC, PHMB or silver nitrate. Both PHMB and silver nitrate displayed combined bacteriostatic and bactericidal activity below the half-maximum inhibitory concentration (IC_{50}). Triclosan in combination with both QSIs displayed antagonistic activity in both UPEC and BSM cells.

Conclusion: PHMB and silver in combination with cinnamaldehyde display synergistic antimicrobial activity in UPEC at non-cytotoxic concentrations, suggesting potential as anti-infective catheter-coating agents.

Introduction

Catheter-associated urinary tract infections (CAUTIs) pose a significant burden to healthcare, accounting for about 80% of

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https://doi.org/10.1016/j.jhin.2023.02.004

healthcare-acquired urinary tract infections (UTIs), with infection rates increasing for each day that the catheter is left in place [1–3]. Uropathogenic *Escherichia coli* (UPEC) are a primary cause of CAUTI and display an array of virulence factors that facilitate the formation of biofilms on the catheter surface and promote colonization of the urinary tract [4,5]. With an ageing global population, the incidence of CAUTI is predicted to rise, posing an escalating risk to the populace and a heightening financial pressure on healthcare service providers [6].

In order to reduce the incidence of CAUTI, approaches to the production of anti-infective catheter surface coatings are being widely considered. Commercially available nitrofurazone-impregnated (ReleaseNF; Rochester Medical Ltd, Lancing, UK) and silver-coated (Lubri-Sil and Bardex IC; Bard Care, Crawley, UK) catheters have been used in clinical settings with mixed outcomes [7,8]. Biocides are a promising antimicrobial agent for use as catheter surface coatings due to their non-specific mechanism of action, working on multiple target sites, so that the selection of resistant bacterial populations is comparatively rare when compared to site-specific therapeutics such as antibiotics [9]. Whereas biocide-impregnated catheter coatings have shown promising antimicrobial activity *in vitro*, growing concerns over biocide cytotoxicity, in addition to reports of inducible biocide resistance and antibiotic cross-resistance, have fuelled the search for new compounds that maintain long-term antimicrobial potency while exhibiting low cytotoxicity [10–12]. One such approach is to use quorum-sensing inhibitors (QSIs) as anti-infective coating agents [13]. Quorum sensing (QS) is a bacterial mechanism of gene regulation in a cell-density-dependent manner. Bacteria release small autoinducer molecules into the surrounding environment, allowing neighbouring bacteria to determine cell density and mediate a group response via synchronized changes in gene expression [14]. Autoinducer-2 (AI-2) is the main QS system used by *E. coli* and has been shown to induce biofilm formation through modulation of a number of motility-associated genes, controlled by motility quorum-sensing regulator MqsR [15].

QS has also been linked to expression of virulence factors across multiple species of bacteria; therefore identification of QSIs has been noted as a potential anti-virulence strategy in antimicrobial chemotherapy [13,15]. *Trans*-cinnamaldehyde has been shown to reduce QS-based activation of virulence factor expression in *Vibrio* species [16,17]. Although the exact mechanism of action in *E. coli* remains unknown, *trans*-cinnamaldehyde has been shown to inhibit the expression of QS-related genes and reduce biofilm formation in UPEC [18–21]. Brominated furanones have also demonstrated an ability to interrupt AI-2-based QS in bacteria and have shown inhibitory effects on *E. coli* biofilms [22,23].

This work aimed to evaluate the pairwise combinatorial effects of the biocides polyhexamethylene biguanide (PHMB), benzalkonium chloride (BAC), silver nitrate, and triclosan in combination with the QSIs *trans*-cinnamaldehyde and (z)-4-bromo-5(bromomethylene]-2(5H)-furanone-C30 (furanone-C30) in UPEC, through determining combined bacteriostatic, bactericidal, and biofilm eradication activities. In parallel, combined cytotoxicity in a BSM cell line was measured. These data enable evaluation of the biocompatibility of these agents for potential use in catheter coatings.

Methods

Bacteria, biocides, and quorum-sensing inhibitors

Six UPEC clinical strains (EC1, EC2, EC11, EC26, EC28, and EC34) previously isolated from urinary tract infections (UTIs) (Stepping Hill Hospital, Stockport, UK) and two laboratory-characterized UPEC stains (EC958 and CFT073) were used in this work [12,24]. Bacteria were cultured in Mueller Hinton broth (MHB) or on Mueller Hinton agar (MHA) (Sigma Aldrich, Poole, UK) and incubated under aerobic conditions at 37 °C overnight unless otherwise stated. BAC (Sigma Aldrich), PHMB (Lonza, Slough, UK), silver nitrate (Alfa Aesar, UK), and *trans*-cinnamaldehyde (Sigma Aldrich) were diluted to working concentrations in water. Triclosan (Sigma Aldrich) and (z)-4-bromo-5(bromomethylene)-2(5H)-furanone) (furanone-C30; which was synthesized in house as described in Guo *et al.* [25]) were diluted to working concentrations in 5% v/v ethanol.

Fractional inhibitory concentrations

Fractional inhibitory concentrations were determined in a checkerboard assay modified from Orhan *et al.* [26]. In brief, two-fold dilutions of QSIs were performed vertically down a 96-well microtitre plate and two-fold dilutions of biocide were performed horizontally in a total volume of 150 μL per well. Overnight UPEC cultures were diluted with MHB to an OD_{600} of 0.008 and 150 μL was added to each test well. Plates were incubated overnight at 37 °C, 100 rpm. The lowest concentration for which growth was completely inhibited was deemed the minimum inhibitory concentration (MIC).

The fractional inhibitory concentration (FIC) is the ratio of the effective concentration of the agent (*a* or *b*) in combination and alone, determined by the equation:

$FIC_a = (MIC_a \text{ in combination})/(MIC_a \text{ on its own})$.

The sum of the FIC_a and FIC_b gives the fractional inhibitory concentration index (FICI). The FICI is defined as synergistic where $FICI \leq 0.5$, additive where $0.5 < FICI \leq 1$, indifferent where $1 < FICI < 2$, and antagonistic where $FICI \geq 2$ [27,28].

Fractional bactericidal concentrations

Following determination of the MIC, 10 μL aliquots were taken from each well of the MIC plate, spot-plated in triplicate on to MHA, and incubated overnight at 37 °C to determine the minimum bactericidal concentration (MBC). Using the MBC values rather than MIC, fractional bactericidal concentration index (FBCI) values were calculated and defined as for FICI.

Fractional biofilm eradication concentrations

Overnight UPEC cultures were diluted to an OD_{600} of 0.008 and 100 μL of culture was added per well to a 96-well plate prior to addition of a peg lid and incubation for 48 h at 37 °C and 30 rpm to allow biofilm formation. Peg lids were then placed into an antimicrobial challenge plate, set out in the same checkerboard format as MIC assays described above. The challenge plate was incubated overnight at 37 °C and 100 rpm. Peg lids were then rinsed twice in 200 μL phosphate-buffered saline (PBS) per peg, placed into a recovery plate containing 200 μL MHB per well and incubated for 72 h at 37 °C and

100 rpm. The MBEC was deemed the lowest concentration that completely inhibited regrowth. The fractional biofilm eradication concentration index was calculated as for the FICI.

Checkerboard MTT assays

Human primary bladder smooth muscle (BSM) cells were cultured in Vascular Cell Basal Medium (ATCC) supplemented with a Vascular Smooth Muscle Cell Growth Kit (ATCC) and incubated at 37 °C and 5% CO_2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT; Sigma Aldrich) was prepared as a stock solution of 50 mg/mL in PBS and filter-sterilized before use. Cytotoxicity of biocides in combination with QSIs was determined in a checkerboard format. BSM cells were seeded at 2.5×10^4 cells per well in a 96-well plate and grown to >80% confluency in 24 h. Antimicrobials were diluted in culture medium and added to the plate, resulting in two-fold dilutions of QSI vertically and two-fold dilutions of biocide horizontally, with a total volume of 200 μL per well. Plates were incubated for 24 h, washed twice in 200 μL PBS and 100 μL of 0.5 mg/mL MTT was added to each well before incubation at 37 °C for 4 h. The MTT containing medium was removed, the precipitated formazan product was solubilized in 200 μL isopropyl alcohol (containing 0.04 M HCl) and incubated at room temperature on an orbital shaker for 1 h. Plates were centrifuged for 5 min at 1000 rpm, 100 μL of supernatant was then decanted and absorbance was measured at 570 nm. All assays were performed in six biological replicates. Survival curves of cytotoxicity data were plotted using a sigmoidal curve of log(inhibitor) vs response with four parameters (variable slope, least squares fit) fit to each single agent, allowing determination of the half-maximum inhibitory concentration (IC_{50}), defined as the concentration at which 50% of the cells survived [29].

Results

Combinatorial antimicrobial effects of biocides and QSIs on UPEC

Fractional inhibitory, bactericidal, and biofilm eradication concentration indices were determined by checkerboard assay against eight UPEC strains (Figure 1; Tables I–III; Supplementary Tables S1–S6). At inhibitory concentrations, cinnamaldehyde and silver nitrate were synergistic against 2/8 strains, EC2 and EC11, and PHMB in combination with furanone-C30 showed synergism against one strain, CFT073 (Figure 1; Table I; Supplementary Tables S1 and S2). Antagonism was observed against 6/8 strains when triclosan was combined with either QSI; in combination with cinnamaldehyde this included EC1, EC11, EC26, EC28, EC34, and EC958; and in combination with furanone-C30 this included EC1, EC2, EC26, EC34, EC958, and CFT073.

At bactericidal concentrations (Figure 1; Table II; Supplementary Tables S3 and S4), cinnamaldehyde in combination with PHMB was synergistic against 6/8 strains (EC1, EC2, EC11, EC26, EC958, and CFT073) and was synergistic in 4/8 strains when combined with silver nitrate (EC11, EC28, EC3, and EC958). Synergism was also observed between furanone-C30 and PHMB against 3/8 strains (EC2, EC11, and EC34) at bactericidal concentrations.

At biofilm eradication concentrations (Figure 1; Table III; Supplementary Tables S5 and S6), PHMB and cinnamaldehyde showed synergism in 6/8 strains (EC1, EC2, EC11, EC26, EC28, and EC958). Synergism was also observed for 5/8 strains for BAC in combination with cinnamaldehyde (EC1, EC11, EC34, EC958, and CFT073) and was observed in all eight strains for silver nitrate in combination with cinnamaldehyde. When combined with furanone-C30, synergism was observed against 3/8 strains for PHMB (EC1, EC28, and EC958), 4/8 strains for BAC (EC1, EC28, EC958, and CFT073) and 6/8 strains for silver nitrate (EC1, EC11, EC26, EC28, EC958, and CFT073).

Cytotoxicity and biocompatibility of combined biocides and QSIs against human bladder smooth muscle cells

Cytotoxicity of biocides and QSIs was determined via MTT assay of primary human bladder smooth muscle cells: the concentrations at which 50% of cells survived (IC_{50}) were determined (Table IV). Concentrations of biocides and QSIs that elicited inhibitory, bactericidal, and biofilm eradication effects against UPEC were compared to concentrations that cause BSM cell cytotoxicity (Tables I–III; Supplementary Tables S1–S6). Both silver nitrate and PHMB in combination with cinnamaldehyde resulted in inhibitory and bactericidal values below the IC_{50}; however, the concentrations required for biofilm eradication (MBEC) were above IC_{50}. Triclosan or BAC in combination with cinnamaldehyde demonstrated antimicrobial activity (MIC, MBC, and MBEC) at concentrations that exceeded the IC_{50}. All biocides in combination with furanone-C30 showed MIC, MBC, and MBEC above the IC_{50} range.

Discussion

Biocides and QSIs demonstrated synergistic, additive and antagonistic interactions at bacteriostatic, bactericidal and biofilm eradication concentrations against UPEC dependent on the test agents combined. Cinnamaldehyde in combination with silver nitrate and furanone-C30 in combination with PHMB demonstrated synergism against 2/8 and 1/8 UPEC strains respectively at inhibitory concentrations. At bactericidal concentrations synergistic interactions were observed between PHMB and cinnamaldehyde or furanone-C30, and between silver nitrate and cinnamaldehyde against 6/8, 3/8, and 4/8 strains, respectively. Synergistic activity was observed at biofilm eradication concentrations where cinnamaldehyde was combined with PHMB, BAC, or silver nitrate against 6/8, 5/8, and 8/8 strains, respectively. Similarly, furanone-C30 demonstrated anti-biofilm synergism with PHMB against 3/8 strains, with BAC against 4/8 strains, and in 6/8 strains when combined with silver nitrate. Cytotoxic combinatorial activity was shown to be dose dependent with bacterial growth inhibitory and bactericidal activity observed below the IC_{50} for PHMB and silver in combination with cinnamaldehyde.

The test biocides and QSIs have a number of growth inhibitory and bactericidal modes of action which may account for the synergism observed at bacteriostatic and bactericidal concentrations. PHMB in combination with cinnamaldehyde was synergistic at bactericidal concentrations against 6/8 UPEC strains. PHMB has been shown to reduce the fluidity of the bacterial membrane due to the bridging of adjacent

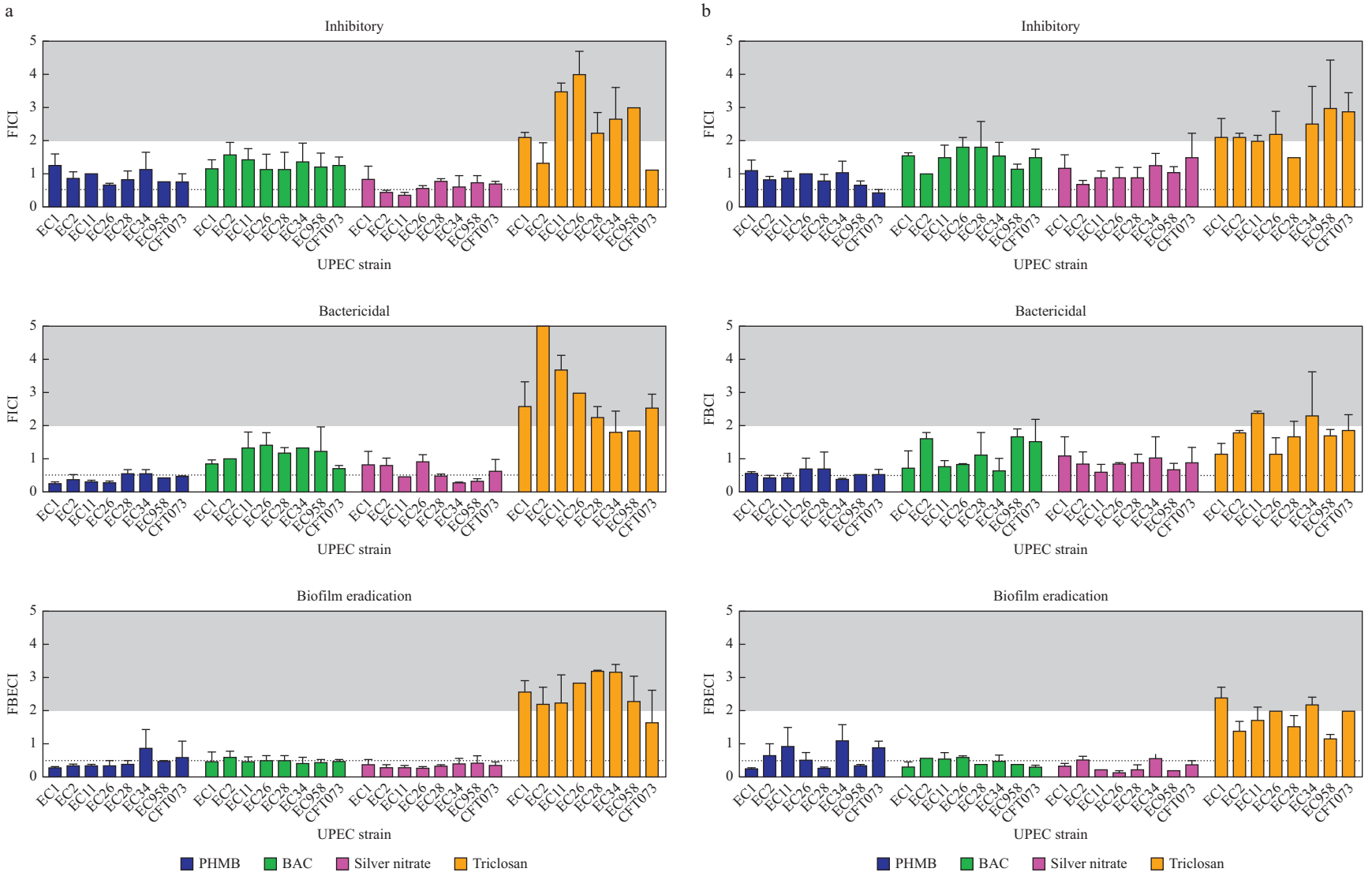

Figure 1. Fractional inhibitory index (FICI) values of biocides, polyhexamethylene biguanide (PHMB), benzalkonium chloride (BAC), silver nitrate, and triclosan in combination with (a) cinnamaldehyde and (b) furanone-C30. All results are an average of two biological replicates, each with three technical repeats. Error bars represent ± 1 standard deviation. Shaded grey area represents antagonistic interactions where fractional concentration indices are >2 and below the dotted line represents synergistic interactions at fractional concentration indices of ≤0.5. UPEC, uropathogenic *Escherichia coli*.

Table I
Minimum inhibitory concentration and fractional inhibitory concentrations of biocides and quorum-sensing inhibitors in uropathogenic *Escherichia coli*

Combination	Independent (μg/mL)				Combined (μg/mL)				FICI
	Biocide		QSI		Biocide		QSI		
	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range
PHMB/Cin	2.6 (0.9)	2–4	526 (199.1)	375–1000	1 (0.2)	0.8–1.3	221.4 (56.7)	166.7–333.3	0.9 (0.2)	0.7–1.3
BAC/Cin	14.2 (1.8)	11.7–15.6	463.5 (60.7)	416.7–583.3	9.4 (2.4)	7.2–13.4	278.7 (61.9)	187.5–375	1.3 (0.2)	1.1–1.6
SN/Cin	22.6 (8.3)	9.8–31.3	484.3 (31)	416.7–500	7.8 (4.6)	3.6–15.6	138 (22.1)	125–187.5	0.6 (0.2)	0.4–0.8
Triclosan/Cin	0.1 (0.1)	0.1–0.3	642.9 (244)	500–1000	0.2 (0.1)	0.1–0.3	500 (221.6)	291.7–1000	2.7 (1)	1.1–4.0
PHMB/F-C30	2.5 (0.7)	1.8–3.7	197.9 (129.9)	125–500	1.1 (0.4)	0.7–2	60.2 (17.4)	28.7–80.7	0.8 (0.2)	0.5–1.1
BAC/F-C30	11.1 (3.5)	7.8–15.6	177.1 (53.4)	125–250	8.3 (2.6)	5.9–14.3	116.5 (34.1)	57.3–166.7	1.5 (0.3)	1–1.8
SN/F-C30	19 (9.2)	7.8–31.3	270.8 (131.3)	125–500	10 (4)	3.9–15.6	124.4 (65.6)	62.5–250	1.1 (0.3)	0.7–1.5
Triclosan/F-C30	0.2 (0.1)	0.1–0.3	224 (32.9)	166.7–250	0.2 (0.1)	0.1–0.4	141.9 (56.5)	93.8–250	2.3 (0.5)	1.5–3

QSI, quorum-sensing inhibitor; FICI, fractional inhibitory concentration index; SD, standard deviation; PHMB, polyhexamethylene biguanide; BAC, benzalkonium chloride; SN, silver nitrate; Cin, cinnamaldehyde; F-C30, furanone-C30.

phospholipids by the biguanide group, causing membrane fissures ultimately leading to leakage of cytoplasmic components [30–32]. It is also known to act as a decoupling agent, disrupting membrane potential and impairing respiration. At bactericidal concentrations, PHMB has been shown to cause direct cell lysis and to condense DNA, halting cell division [30–32]. Cinnamaldehyde has also been shown to permeabilize the cell membrane, to inhibit FtsZ polymerization thus impairing cell division, and to reduce ATPase activity [33,34]. The combination of two membrane-active agents may exert a cumulative disruptive effect to the bacterial cytoplasmic membrane, explaining the synergistic growth inhibitory and bactericidal activity. Furthermore, increased cellular permeability would facilitate entry of the active agents into the cytoplasm, enabling them to reach intracellular targets efficiently. The combined targeting of cellular replication through condensation of DNA and inhibition of FtsZ polymerizations could also contribute towards the bacteriostatic synergism observed.

Silver nitrate has been shown to inactivate microbial enzymes, such as NADH-dependent dehydrogenases, through interaction with thiol groups leading to dysregulation of membrane potential and impaired respiratory capacity, impacting growth and replication. It is also suggested to cause cell envelope damage due to membrane shrinkage and condensation of DNA [35–38]. The combination of inhibited ATPase activity by cinnamaldehyde and further inhibited activity of respiratory enzymes by silver nitrate could contribute towards the synergistic growth inhibitory activity observed in UPEC, whereas synergistic bactericidal effects may be attributed to combined disruption of the cell envelope and underlying cytoplasmic membrane [33].

PHMB and furanone-C30 in combination showed synergistic capability at bacteriostatic and bactericidal concentrations. Furanones have been shown to increase membrane permeability and affect the membrane potential of *Pseudomonas aeruginosa*, although its membrane-disrupting effects in *E. coli* have not been previously documented [39]. Any increase in membrane permeability due to the actions of furanone-C30 may increase the intracellular accessibility of PHMB, facilitating contact with intracellular targets, such as DNA. Furthermore, PHMB is a known decoupling agent — thus, when paired with further disruption to membrane potential it could impair respiration in a cumulative fashion and thus cell growth.

Triclosan was antagonistic in bacteriostatic and bactericidal assays when combined with both cinnamaldehyde and furanone-C30. Within *E. coli*, triclosan inhibits enoyl acyl carrier protein reductase enzyme FabI, inhibiting fatty acid synthesis at inhibitory concentrations and causing membrane damage at bactericidal concentrations [40–42]. At sub-inhibitory concentrations, triclosan has been shown to induce oxidative stress, to cause damage to the membrane, and to induce expression of genes involved in the regulation of the SOS response [43]. Exposure of *E. coli* to sub-lethal concentrations of triclosan has been shown to induce tolerance to antibiotics through induction of ppGpp synthesis [44]. Studies have indicated that elevated intracellular levels of ppGpp activate the bacterial toxin–antitoxin molecule TA, resulting in the bacterial cell entering a persister-like state with decreased overall antimicrobial susceptibility [45,46].

Table II
Minimum bactericidal concentration and fractional bactericidal concentrations of biocides and quorum-sensing inhibitors in uropathogenic *Escherichia coli*

Combination	Independent (μg/mL)				Combined (μg/mL)				FICI
	Biocide		QSI		Biocide		QSI		
	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range
PHMB/Cin	7.7 (3.9)	4—14	1625 (688.63)	1000—3000	1.3 (0.4)	0.9—2	283.9 (64.9)	166.67—333.33	0.4 (0.12)	0.3—0.6
BAC/Cin	18 (4)	14.3—26.4	1291.7 (292.1)	1000—1666.7	9.8 (3.3)	4.6—15.6	599 (309.4)	125—1083.3	1.1 (0.3)	0.7—1.4
SN/Cin	29.2 (12.1)	14.97—52.08	1343.8 (328.7)	833.3—1833.3	10.2 (4.3)	3.9—18.9	218.8 (62)	145.8—291.7	0.58 (0.2)	0.3—0.9
Triclosan/Cin	2.7 (1.4)	01—4.7	1895.8 (234.7)	1333.3—2000	5.4 (2.7)	2.8—9.3	1291.7 (452.1)	1000—2000	4.3 (4.9)	1.8—16.4
PHMB/F-C30	11 (4.9)	6.3—21.3	268.2 (105.3)	125—500	2.2 (1.6)	0.3—5	90.2 (33)	44.3—130.2	0.6 (0.1)	0.4—0.7
BAC/F-C30	21.2 (7.1)	10.4—33.9	234.4 (42.8)	145.8—291.7	9.7 (2.8)	7.2—15.63	126.6 (56.4)	73—218.8	1.1 (0.4)	0.6—1.7
SN/F-C30	28.3 (12.6)	11.7—52.1	328.1 (136.3)	125—500	84.3 (202.8)	5.9—586	113.9 (35.6)	57.3—156.3	0.9 (0.2)	0.6—1.1
Triclosan/F-C30	3.9 (2.07)	1.3—6.7	322.9 (101.5)	250—500	3.7 (2.1)	1.38—6	237 (100.2)	125—416.7	1.8 (0.5)	1.2—2.4

QSI, quorum-sensing inhibitor; FICI, fractional inhibitory concentration index; SD, standard deviation; PHMB, polyhexamethylene biguanide; BAC, benzalkonium chloride; SN, silver nitrate; Cin, cinnamaldehyde; F-C30, furanone-C30.

At biofilm eradication concentrations, combinations of PHMB, BAC, and silver nitrate with cinnamaldehyde demonstrated synergism against 6/8, 5/8, and 8/8 UPEC strains, respectively, and combined with furanone-C30 demonstrated synergy against 3/8, 4/8, and 6/8 strains, respectively. Both cinnamaldehyde and furanone-C30 are proposed to inhibit quorum-sensing-related genes and biofilm formation in *E. coli*. Cinnamaldehyde has been shown to inhibit biofilm formation in UPEC strains and to reduce expression of attachment associated genes including *fimA*, *fimH*, *focA*, *sfaA*, *sfaS*, and *papG*, suggesting a potential mechanism to interfere with adhesin expression, thus attachment and initiation of biofilm formation [19—21]. Furthermore, reduced expression of AI-2-associated promoters in *Vibrio harveyii* has been observed following exposure to cinnamaldehyde, and AI-2 signalling has been associated with biofilm formation of *E. coli* through AI-2 control of the *mqsR* motility regulator [15,18]. Furanone-C30 has also been demonstrated to interrupt the AI-2 signalling of *E. coli*, reducing the expression of genes associated with chemotaxis, motility, and flagellar synthesis, and to inhibit biofilm formation [23,47]. Both cinnamaldehyde and furanone-C30 could therefore be interrupting biofilm initiation and development and the remaining residual bacteria may then be eliminated more readily by lower concentrations of biocide than would be required to eradicate a mature biofilm.

To produce an effective anti-infective catheter coating with a high level of biocompatibility we ideally require synergistic antimicrobial activity while avoiding synergistic cytotoxic activity. Effective antimicrobial concentrations against a panel of UPEC were compared to the cytotoxicity of the agents against a BSM cell line. The MIC and MBC for combinations of PHMB or silver nitrate with cinnamaldehyde were below the IC_{50} range whereas the MBEC was above. All other combinations of biocides and QSIs showed bacteriostatic, bactericidal, or biofilm eradication activity at concentrations exceeding IC_{50}.

Both PHMB and cinnamaldehyde have membrane permeabilizing activity against both bacteria and eukaryotic cells, which may increase the intracellular cytotoxic effects of these agents [32,33]. However, PHMB is less readily attracted to the relatively neutrally charged mammalian cell membrane when compared to the electronegative bacterial cell, and, whereas PHMB can enter both eukaryotic and bacterial cells, the condensation of DNA has been shown to impact only bacteria as it does not enter the eukaryotic nucleus [30,31]. Cinnamaldehyde has demonstrated cytotoxic effects which include induction of apoptosis and decreases in mitochondrial membrane potential [48]. The combination of PHMB and cinnamaldehyde could increase the permeability of the membrane and allow more of the biocides into the bacterial cell, facilitating access to intracellular targets, such as DNA. There would be lower cytotoxic impacts due to the selective mode of action of the biocides towards the bacterial cell and inability of PHMB to enter the nucleus.

BAC and cinnamaldehyde in combination led to enhanced cytotoxic activity compared to the agents used independently with inhibitory, bactericidal or antibiofilm activity observed above IC_{50}. BAC and cinnamaldehyde both have membrane permeabilizing activity; however, BAC, like PHMB, is cationic and is more readily attracted to the bacterial membrane [30]. In addition to membrane permeabilization, BAC has been shown to induce apoptosis and necrosis in human cells [49—51]. The complementation of mechanisms between

Table III
Minimum biofilm eradication concentrations and fractional biofilm eradication concentrations of biocides and quorum-sensing inhibitors in uropathogenic *Escherichia coli*

Combination	Independent (μg/mL)				Combined (μg/mL)				FICI
	Biocide		QSI		Biocide		QSI		
	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range
PHMB/Cin	116.2 (29.6)	78.1–166.7	1854.2 (392.8)	1166.7–2333.3	20.1 (13.6)	6.5–41.7	460.9 (90.8)	270.8–583.3	0.5 (0.2)	0.3–0.9
BAC/Cin	120.5 (47.7)	46.9–197.9	1645.8 (207.7)	1333.3–2000	22.9 (9.4)	11.7–33.8	466.2 (93.1)	312.5–583.3	0.5 (0.1)	0.4–0.6
SN/Cin	2324.2 (886.1)	520.8–3333.3	1583.3 (356.4)	1000–2000	278.7 (223)	41.7–781.3	354.2 (115.2)	229.2–583.3	0.3 (0.1)	0.2–0.4
Triclosan/Cin	5.5 (3.8)	1.7–13.3	2041.7 (1143.5)	1000–4666.7	9.3 (5.5)	2.3–17.3	1500 (671.1)	583.3–2833.3	2.5 (0.5)	1.7–3.2
PHMB/F-C30	159.8 (93)	44.3–312.5	295.6 (151.3)	125–500	34.6 (28.1)	5.9–83.3	81.7 (38)	26–125	0.6 (0.3)	0.3–1.1
BAC/F-C30	96.4 (42.6)	41.7–177.1	307.3 (138.5)	125–500	21.4 (9.3)	12.37–33.9	59.6 (17)	36.5–93.8	0.5 (0.1)	0.3–0.6
SN/F-C30	3374.4 (1503.6)	1380.2–5833.3	260.4 (96.4)	125–416.7	346.8 (314.1)	52.1–925.8	58.9 (30.1)	15.6–114.6	0.3 (0.2)	0.2–0.6
Triclosan/F-C30	4.1 (3.1)	0.8–8	294.3 (111.6)	125–458.3	3.5 (2.6)	0.7–8	231.8 (49.1)	125–291.7	1.8 (0.4)	1.2–2.4

QSI, quorum-sensing inhibitor; FICI, fractional inhibitory concentration index; SD, standard deviation; PHMB, polyhexamethylene biguanide; BAC, benzalkonium chloride; SN, silver nitrate; Cin, cinnamaldehyde; F-C30, furanone-C30.

Table IV
Half-maximum inhibitory concentrations (IC_{50}) of biocides and quorum-sensing inhibitors against bladder smooth muscle cells

Combination	IC_{50} (μg/mL)	
	Biocide	QSI
PHMB/Cin	10.9 (7.6–15.7)	1574 (906.4–2735)
BAC/Cin	3.3 (2.7–4)	7.6 (0.01–4504)
SN/Cin	17.3 (14.3–20.8)	675.2 (546.8–833.7)
Triclosan/Cin	16.7 (8.4–33)	234.4 (188.2–292.1)
PHMB/F-C30	5.3 (4.7–6)	6.7 (4.1–10.9)
BAC/F-C30	1.9 (0.9–4)	15.2 (12.5–18.5)
SN/F-C30	5.4 (1.5–19.8)	17.1 (13.5–21.5)
Triclosan/F-C30	9.8 (7.6–12.6)	21.6 (12.9–36.1)

QSI, quorum-sensing inhibitor; FICI, fractional inhibitory concentration index; SD, standard deviation; PHMB, polyhexamethylene biguanide; BAC, benzalkonium chloride; SN, silver nitrate; Cin, cinnamaldehyde; furanone-C30 (F-C30).
Data are average IC_{50} and 95% confidence intervals in parentheses ($N = 6$).

cinnamaldehyde and BAC against mammalian cells may account for the low concentrations required for cytotoxic effects.

Silver nitrate in combination with cinnamaldehyde demonstrated enhanced cytotoxic activity compared to the agents used independently. MIC and MBC of the combined agents in UPEC fell below IC_{50} whereas the MBEC was above. Silver nitrate has multiple targets against both bacterial and mammalian cells in addition to causing membrane permeabilization. The permeabilization of the cell membrane by both cinnamaldehyde and silver may contribute to cumulative cytotoxic effects at concentrations required to eradicate bacterial biofilms [36,37].

Combinations of triclosan and cinnamaldehyde had antagonistic effects on both bacteria and BSM cells when compared to the agents used independently. The effective antimicrobial concentrations all fell above the IC_{50} values. Triclosan has been shown to be cytotoxic through impairment of mitochondrial activity, loss of membrane stability, and is thought to lead to direct cell apoptosis [52]. Both triclosan and cinnamaldehyde have poor solubility; the combination of both agents may antagonize this effect and lead to poor bioavailability of the active agents, hence the antagonistic effects observed in both cytotoxicity and antimicrobial assays.

All cytotoxic concentrations of furanone-C30 were below those required for antimicrobial activity when combined with all four biocides. Furanones have been shown to be cytotoxic and as such they are often a focus of anti-cancer drug discovery [53]. Efforts have been made to reduce the toxicity of the naturally occurring furanones and derivatives including furanone-C30 [54]. However, the exact mechanism of furanone-C30 toxicity against mammalian cells is currently unknown.

In conclusion, existing anti-infective catheter coatings often use a single biocidal agent in an attempt to prevent bacterial colonization of the catheter surface. This study investigated the combinatorial potential of biocides and QSIs in impairing UPEC growth and biofilm establishment in parallel with evaluating cytotoxicity against bladder smooth muscle cells. Promising combinations of cinnamaldehyde with either

PHMB or silver show active antimicrobial synergism against UPEC below cytotoxic concentrations, suggesting potential as an anti-infective catheter coating.

Conflict of interest statement
None declared.

Funding sources
This research was funded by The Healthcare Infection Society and Biomolecular Sciences Research Centre at Sheffield Hallam University. The HIS grant ID is SRG 2019 09 001.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2023.02.004.

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