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Investigation of Alternative Biocidal Options against Foodborne Multidrug Resistant Pathogens

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Abstract

Foodborne diseases represent a significant threat to public health safety, with the European Centre for Disease Prevention and Control (ECDC) reporting *Campylobacter*, *Listeria* and Enterobacteriaceae, primarily *E. coli* and *Salmonella*, as the most frequent cause of illness. At the same time the waged war against antibiotic resistant superbugs continues, where patient cases are becoming more prevalent and medically difficult to treat, as pathogens acquire different combination mechanisms to resist conventional antimicrobial therapy. Antimicrobial resistance arises from the misuse of antibiotics, where their prolonged use in the medical sector exerts selective pressure on bacteria. However, a lesser known route lies within the food chain where MDR bacteria can be derived from food-producing animals or from cross-contamination during food processing. To mitigate disease incidence, the EU currently endorse a "fork to farm" approach, highlighting the necessity for prevention and control measures throughout the entire food chain, including safe handling by the consumers themselves. Biocides play a critical role in limiting the spread of infectious disease. Indeed, the food industry is dependent on these agents, where risk of toxic residual levels in foodstuffs and reduced susceptibility to biocides (namely that of QACs and chlorine), coupled with the potential for the development of a phenotype of cross-resistance to clinically important antimicrobials are prominent causes for concern. Studies conducted investigate the activity of two novel disinfectant compounds, peracetic acid, triameen in comparison to BAC against a variety of foodborne pathogens. Findings suggest that both novel test agents may be suitable disinfectants for use in food, agricultural, and veterinary areas, where all MDR species were susceptible to treatment. In particular, peracetic acid offers promising prospects, being further capable of inactivating *Bacillus* spores while concomitantly being an eco-friendlier alternative.

Keywords: Foodborne; Enterobacteriaceae; *Listeria*; *Bacillus*; Resistance; Susceptibility

Introduction

An adequate supply of nutritious food is essential for maintaining a healthy balanced diet, ensuring optimal growth and development and disease prevention. As the population expands there is an increasing demand for food sources to support a varied diet. Furthermore, as increasing numbers of people are moving towards a non-processed fresh food diet there is an increase consumption of fruit and vegetable produce globally. Consequently, there is also an increase in foodborne disease relating to microbial pathogens. While foodborne disease is often self-limiting and acute by nature, in those with compromised immunity, old and young people, infection can be chronic, severe and require hospitalization. Contamination of food can occur at all stages of food production from pre-harvest, harvest and post-harvest preparation. Contamination at pre-harvest may be related to soil, fertilizing manure, bio solids and water supplies [1] and is influenced by seasonality, weather, temperature, and solar exposure. Contamination at harvest and pre-harvest relates to environmental cleanliness, animal housing, faecal transmission at animal slaughter and human food handling. Bacterial virulence factors also play a role in their survivability, pathogenicity, and resistance to food processing methods. *Escherichia coli* and *Listeria monocytogenes* for example are known to survive colder weather climates with *L. monocytogenes* capable of growth at refrigeration temperatures. Indeed, listeriosis caused by *L. monocytogenes* (of which 99% of cases are foodborne) has a mortality rate of 30% with fetal abnormalities and abortions also an issue [2]. Certain foodborne thermoduric pathogenic species including emetic and enterotoxin producing *Bacillus cereus* and *Bacillus subtilis* are known to possess spore formation abilities making them very heat and chemical resistant whereas others such as *Staphylococcus aureus* produce heat-resistant toxins associated with foodborne disease [3]. Enterobacteriaceae, including *E. coli*, *Klebsiella*, *Proteus* and *Salmonella* species which are enteric Gram-negative pathogens are frequently

associated with faecal contaminated meat, meat products and dairy foodstuffs including cheeses, infant milk formulas and milk powder. Many of these species possess intrinsic and acquired antibiotic resistance and are often Multidrug Resistant (MDR). MDR isolates of Gram-negative *Enterobacter*, *Klebsiella*, *E. coli*, *Proteus* species and *Pseudomonas aeruginosa* have been detected in numerous food items [4]. Psychrotrophic biofilm-forming strains of *Pseudomonas* have become a major contributor to foodborne contamination and contamination off food production environments [5]. Additionally, *P. aeruginosa* is notoriously MDR and is listed as top priority on the World Health Organisation (WHO) priority pathogen list. The presence of *Enterococcus* species in food from water and faecal contamination or intentionally as probiotics has also come under question as many species are opportunistic pathogens associated with nosocomial infections, antibiotic resistance particularly to vancomycin and MDR [6]. Indeed, *Enterococcus* species possess intrinsic resistance to cephalosporins, sulphonamides, lincosamides, β-lactams, and aminoglycosides with acquired resistance to many drugs also evident. Foodborne Campylobacteriosis is predominately associated with *Campylobacter jejuni* a Gram-negative bacterium associated with contaminated poultry meat and eggs. *C. jejuni* tends to cause a more severe infection with bloody diarrhoea often evident; furthermore, *C. jejuni* bacteraemia during pregnancy can cause intrauterine infection of the foetus, abortion or stillbirth, with vertical transmission to the infant potentially causing neonatal death, enteritis, bacteraemia, and/or meningitis [7]. In terms of food and consumer safety, the best measures to prevent foodborne disease with these robust, recalcitrant species are prevention of contamination with adequate disinfection at all stages of production. Disinfection options for food production include Quaternary Ammonia Compounds (QACs) Dodecyldimethylammonium chloride (DDAC) and Benzalkonium Chloride (BAC), hypochlorite's, iodophors and chlorine dioxide-based solutions. Biocidal efficacy for these disinfectants is associated with numerous variables including the microbial load, presence of organic matter, contact time, temperature, and pH. More recently issues relating to residual levels of toxic disinfection solutions such as QACs and chlorines in foodstuff have become prominent. As such, there is a growing need for alternative environmentally friendly disinfection options. It has also become evident that exposure of microorganisms to sub lethal concentrations of biocidal disinfections such as QACs and chlorine promotes the emergence of disinfectant resistant bacterial species, particularly evident in MDR isolates showing cross resistance to disinfectants and antibiotic drug therapy. In the context of assessing novel disinfection options for food production environments at pre-harvest and harvest the aim of this study is to determine the efficacy of peracetic acid and triameen in comparison to BAC against a range of MDR foodborne pathogens. Peracetic acid is a powerful oxidant capable of oxidising the outer cell membranes of micro-organisms, inducing biocidal activity. Triameen is a fatty amine derivative and a highly effective antimicrobial agent. Benzalkonium Chloride (BAC) belongs to the class of Quaternary Ammonium Compounds (QACs), where studies suggest their ubiquitous use in commercial products can generate selective pressure within environments, promoting cross resistance among microbes to a variety of compounds and a move away from this compound in food production environments.

Materials and Methods

Common foodborne and clinical pathogens including Gram positive *L. monocytogenes*, *S. aureus*, *B. cereus*, *B. subtilis* and *E. hirae* and Gram negative *E. coli*, *S. typhimurium*, *C. jejuni* (33560), *P. aeruginosa*, *P. vulgaris* (13315) and *K. pneumonia* were selected for testing. Quality control ATCC strains of *E. coli* (25922), *P. aeruginosa* (10662), *E. hirae* (10541), *S. aureus* (25293), *B. cereus* (11778) and *B. subtilis* (23857) were also subject to testing for comparative sensitivity analysis to isolated species.

Bacterial isolation, identification, culture, and maintenance

Isolated bacterial species from agricultural animal cases of infection, were inoculated in nutrient broth (Cruinn Diagnostics, Dublin, Ireland) and incubated at 37°C for up to 24 hours before streaking onto nutrient agar (Cruinn Diagnostics, Dublin, Ireland). Individual colonies were re-streaked for isolation and pure isolated colonies inoculated into nutrient broth for further biochemical characterization. Colonies were identified based on their morphological characteristics, biochemical profile and growth on selective agars, specifically CHROMagar™ MRSA, CHROMagar™ *Pseudomonas* (CHROMagar, Paris), Harlequin™ *E. coli*/Coliform Medium, PEMBA *Bacillus cereus* Medium (Cruinn Diagnostics, Dublin, Ireland), Harlequin™ *Listeria* Chromogenic Agar, Baird Parker agar (LabM, Cruinn Diagnostics, Dublin, Ireland) and *Klebsiella* Chromo Select Selective (Sigma Aldrich, Dublin, Ireland). Identity was confirmed via colony Polymerase Chain Reaction (PCR). Specifically, single colonies of each bacterial isolate were subcultured in nutrient broth and incubated overnight at 37°C. Genomic DNA was directly extracted using the GenElute ™ Bacterial Genomic kit (Sigma, Ireland) according to the manufacturer's instructions and bacterial primers ITS_8F 5'-AGGTTTGATCCTGGCTCAG-3' and ITS_U1492R 5'-GGTTACCTTGTTACGACTT-3' (Sigma Aldrich, Dublin, Ireland) used for amplification of 16s rRNA gene. PCR was performed in a total reaction volume of 20 µl, containing 17 µl red Taq 1.1 x master mix (VWR, Dublin, Ireland) 1 µl UniF, 1 µl UniR and 1 µl of pure genomic DNA eluate. DNA amplification was performed in a thermo cycler (VWR, Dublin, Ireland) using the recommended parameters. Clean-up and gene sequencing of PCR products was completed by Source Bioscience (Waterford, Ireland). Strains were stored and cultured in nutrient broth/agar at 37°C and identity confirmed via Gram stain prior to each experimental set up.

Antibiotic resistance profile

Antibiotic resistance profiles were established using selective agars (CHROMagar™ ESBL, Harlequin™ O.R.S.I.M. [Oxacillin Resistant Staphylococci Isolation Medium]) (Cruinn

Diagnostic, Ireland) and a range of antibiotic susceptibility disks (ThermoFisher Scientific, Ireland) according to the European Committee for Antibiotic Susceptibility Testing (EUCAST) recommendations. Specifically, 100 µL of ca. 1 × 10^6 cfu/ml of a 6 hour bacterial culture was overlaid on to Muller-Hinton agar plates absent of surface moisture (EUCAST, 2020). An antibiotic inoculated disk was placed in the centre of the plate and incubated inverted for 24 hours at 37°C. Zones of inhibition were measured in millimetre (mm) where the absence of a zone of inhibition denotes resistance in the organism. Species were then graded as Susceptible, standard dosing regimen (S) or susceptible, Increased exposure (I) or Resistant (R) according to EUCAST. A resistance profile was established in accordance with the WHO priority pathogen list for each species under investigation **(Table 1)**.

Novel biocidal agents

The antibacterial agents investigated in this study are pure biocides used as disinfectants alone or in commercial brands. The concentrations described are the concentration of the active component and include the concentration used following manufactures instructions. In the present study, the activity of each biocide will be determined against multidrug resistant pathogens capable of causing foodborne disease and severe clinical illness.

Kirby Bauer assay

The Kirby Bauer assay was carried out to determine the effect of disinfectants on microbial species with the presence and absence of an interfering substance. The bacterial organisms were grown on Mueller-Hinton (Criuinn Diagnostics) agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit the test organism. Agars were prepared as per manufacturer's instructions in deionized water at room temperature and poured to a depth of 4 mm. Immediately before inoculation, media was checked to ensure it was moist but free of water droplets on the agar surface and the Petri dish lids. The test inoculum was prepared from a pure culture grown in nutrient broth for 6 hours. To determine the effect of an interfering substance, 1 mL of ca. 1 × 10^7 microbial cells was added to 9 mL Bovine Serum Albumin (BSA) to give a working microbial count of 10^6 cells in 3 g/L and 10 g/L BSA solution. Subsequently, 100 µL 10^6 cells/mL microbial suspensions were transferred onto replicate agar plates and spread with a sterile L-shaped spreader (Cruinn Diagnostics) to ensure even disruption across the agar surface. Filter disks (6 mm) were immersed in the test solutions at concentrations of 0.01%, 0.1% and 1% (v/v) peracetic acid and 0.01%, 0.1% and 1% (v/v) triameen and BAC for 15 seconds where excess solution was allowed to drip off the disk. Subsequently, the disk was placed in the centre of the inoculated plate. Plates were then inverted and incubated for 24 hours at 37°C. Zones of inhibition were then measured using a Vernier calliper in millimetres as per Meade et al. for each test chemical and each test organism [8].

Quantitative suspension test

Suspension tests were conducted in accordance to the guidelines of the BS EN 1656 for antibacterial testing of chemical disinfectants that form a homogeneous physically stable preparation in hard water for use in food, agricultural, and veterinary areas. For compliance with test method, test chemicals must achieve a 10^5 bacterial cell reduction in treatment times less than 30 minutes, where test organisms include *P. aeruginosa*, *P. vulgaris*, *E. hirae*, *S. aureus* and *L. monocytogenes*. Bacterial test suspensions were prepared by seeding sterile nutrient broth with an isolated colony and incubating under rotary conditions (125 rpm) for 12 hours at 37°C. Cell counts were adjusted to 10^9 cfu/mL with sterile PBS. Chemical test solutions were prepared as per manufacture instructions for use on site and at a concentration above and below this working concentration giving a range of 0.05%, 0.1% and 1% (v/v) peracetic acid and 0.01%, 0.1% and 1% (v/v) triameen and BAC. Prior to testing all reagents are equilibrated to the test temperature of 10°C using a water bath. Subsequently 8 mL of the test product was transferred to a sterile container with 1 mL of sterile water. Afterward, 1 mL of microbial suspension containing 1 × 10^9 bacterial cells was added. Additionally, 1 mL of interfering substance at 3.0 g/L BSA and 10 g/L BSA with was added with subsequent incubation for 0-15 minutes with mixing in a 10°C water bath. At set intervals of 5 minutes, 10 minutes and 15 minutes, 1 mL of the test mixture was transferred into a tube containing 8 mL neutralizer (30 g/L polysorbate 80+3 g/L lecithine/l-a-phosphatidylcholine from egg yolk) (Sigma, Ireland) and 1mL of sterile water. Samples were mixed and incubated in the water bath for 5 minutes. After neutralization 100 µL of this bacterial suspension was transferred onto agar plates in triplicate and incubated at 37°C for 24 hours. Surviving cells of treated organisms was counted to determine the level of bacterial inactivation following exposure to the test solutions compared to the untreated control (PBS).

Anti-sporicidal activity

A quantitative suspension test for the evaluation of sporicidal activity of chemical disinfectants used in food, industrial, domestic, and institutional areas was used in accordance with BS EN 13704. *Bacillus subtilis* and *Bacillus cereus* endospores were produced per the method of Garvey et al. and stored at -20°C when not in use [9]. For compliance with this test, test chemicals must achieve a 10^4 cfu/mL reduction of spores in treatment times less than 60 minutes in the presence of an interfering substance at 20°C. Test chemical under study included peracetic acid and triameen at concentrations of 2.5% and 5% (v/v) respectively. Specifically, 1 mL of a 10^8 cfu/mL spore suspension was added to 8 mL of the test chemical with 1 mL 3.0 g/L BSA and allowed to react for up to 60 minutes at 20°C. Following varying treatment time points, the reaction was neutralised as previously described and 100 µL samples were spread on nutrient agar plates in triplicate and incubated at 37°C for 24 hours to establish a viable cell count.

Statistics

All the experiments were performed three times with three plate replicates for each experimental data point providing a mean result for each test species and antimicrobial susceptibility (+/-standard deviation). The $\log_{10}$ inhibition of growth was calculated as the $\log_{10}$ of the ratio of the concentration (cfu/ml) of the non-treated (N0) and treated (N) samples [$\log_{10}$ (N0/N)]. Student T tests were conducted to determine significance levels ($p<0.05$) of microbial susceptibility to treatment using Minitab 16 (Minitab Ltd, Coventry, UK).

Results

Table 1 displays the resistance profile of test species to a range of antibiotics, where ATCC strains show significant more susceptibility than their isolated counterparts. For instance, WHO critically important *E. coli* (isolate) shows resistance to Streptomycin, Chloramphenicol, Ampicillin and Cefpodoxime, while in comparison the ATCC strain exhibits susceptibility, producing zones of 20 mm, 28 mm, 15 mm and 30 mm respectively. Both strains show resistance to Vancomycin, where erythromycin proves just as inadequate, only providing a zone of 10 mm for *E. coli* (isolate), while the ATCC strain shows resistance. Moreover, WHO critically important *P. aeruginosa* (isolate) demonstrates clear resistance to all tested antibiotics, where in comparison its ATCC counterpart was susceptible to Chloramphenicol and Erythromycin producing zones of 19 mm and 22 mm respectively. However more marginal zones are seen for the Aminoglycosides and Cephalosporins, where zones of 7 mm, 10 mm and 13 mm were produced by the ATCC strain for Kanamycin, Streptomycin and Cefpodoxime respectively. Further resistance to the Penicillin and Glycopeptide classes is also evident. *K. pneumonia*, another notoriously challenging pathogen, showed resistance to all tested antibiotics, having a negligible zone of 13 mm for Kanamycin and 18 mm for Cefpodoxime, failing to meet the EUCAST target zone of 21 mm. The *Proteus vulgaris* species and high priority *S. typhimurium* and *C. jejuni* were susceptible to the Aminoglycosides, Chloramphenicol and Cephalosporins, though notably, *S. typhimurium* showed less sensitivity to Streptomycin, having a zone of just 11 mm. Moreover, *S. typhimurium* and *P. vulgaris* show resistance to Vancomycin, Erythromycin and Ampicillin; while on the other hand, *C. jejuni* remained susceptible, producing zones of 29 mm, 35 mm and 34 mm respectively. All *Bacillus* species were resistant to the Penicillin's (Ampicillin), where isolated counterparts of *B. cereus* and *B. subtilis* showed further resistance to 3rd generation Cephalosporins (Cefpodoxime). Moreover, both isolates showed reduced sensitivity to Vancomycin, producing a respective zone of 12 mm, while corresponding zones of 16 mm and 24 mm can be seen for ATCC *B. cereus* and *B. subtilis*. However, high levels of susceptibility for Aminoglycosides, Chloramphenicol and Macrolides is evident, particularly for that of *B. subtills* (ATCC), where zones range from 29 mm-35 mm. Chloramphenicol and Kanamycin proved effective against *L. monocytogenes*, however lower levels of sensitivity can be seen for Streptomycin, Vancomycin and Cefpodoxime, with respective zones of 11 mm, 13 mm and 10 mm produced. Furthermore, *L. monocytogenes* failed to meet EUCAST target zones of 21 mm and 16 mm for Erythromycin and Ampicillin, with zones of 15 mm and 10 mm represented resistance. ATCC *E. hirae* remained susceptible to all antibiotics, though notably lower levels of sensitivity can be seen for the Aminoglycosides and Penicillin, producing marginal zones of 14 mm and 13 mm respectively. In comparison, the isolated counterpart showed a greater zone (20 mm) for Ampicillin, with its intrinsic resistance to Cephalosporins, Aminoglycosides and Vancomycin evident. However, the *E. hirae* species do share similar sensitivity patterns for both Chloramphenicol and Erythromycin, with zones >21 mm. Chloramphenicol provided the greatest levels of inhibition for both *Staphylococcus* species, however the isolated counterpart proved to be more resilient, displaying resistance to Streptomycin, Erythromycin, Ampicillin and Cefpodoxime as well as being a Methicillin Resistant (MR) strain, characterised by its growth on Harlequin™ O.R.S.I.M agar.

Table 1: Antibiotic profile of test species to a range of antibiotics from varying drug classes with EUCAST target zones given for species for which such information is available.

Drug Class		Aminoglycoside		Glycopeptide	Chloramphenicol	Macrolide	Penicillin	Cephalosporins
Antibiotic		Kanamycin	Streptomycin	Vancomycin	Chloramphenicol	Erythromycin	Ampicillin	Cefpodoxime
Concentration (µg/disc)		30	10	30	30	15	10	10
E. coli	ATCC	18	20	R	28 (S)	R	15	30
*E. coli**	Isolate	12	R	R	R [17]	10	R [14]	15 (R)
P. aeruginosa	ATCC	7	10	R	19	22	R	13
*P. aeruginosa**	Isolate	R	R	R	R	R	R	R
*K. pneumonia**	Isolate	13	R	R	R [17]	R	R [14]	18 (R)
P. vulgaris	ATCC	23	20	R	25	R	R	31

*Salmonella***	ATCC	19	11	R	22	7	7	24
*C. jejuni***	ATCC	19	22	19	29	35 (S)	34	22
B. cereus	ATCC	18	20	16	22	23	R	14
B. cereus	Isolate	18	20	12	26	21	R	R
B. subtilis	ATCC	29	30	24	35	31	R	19
B. subtilis	Isolate	22	20	12	26	21	R	R
L. monocytogenes	Isolate	19	11	13	27	15 (R)	10 (R) [16]	10
E. hirae	ATCC	14	14	16	26	21	13	17
E. hirae	Isolate	10	R	R	23	26	20	R
S. aureus	ATCC	15	13	14	25	20	23	19
*MR S. aureus***	Isolate	19	R	16	22	9 (R) [18]	R	R

* WHO critically important pathogen; ** WHO high priority pathogen; target zones for antibiotic against listed strain according to EUCAST; R denotes Resistance; I denotes Intermediate Susceptibility; and S denotes Susceptibility to antibiotic drug

Table 2 depicts the activity of novel disinfectant agents, peracetic acid, triameen and BAC, where various formulations are regularly used in food and medical sector. All three agents provided high levels of bacterial inhibition, although to varying degrees. In terms of susceptibility, Gram-negative species display the highest levels of sensitivity to peracetic acid at a concentration of 1%, with the exception of *S. typhimurium* where triameen was most effective providing a zone of 30 mm. In addition, for Gram-negative species BAC continuously remained to be the least effective of the three disinfectants, specifically at the lower concentration of 0.01%, where *C. jejun*i appeared to be the only organism in which a sufficient zone (14.5 mm) was provided. Indeed, *P. aeruginosa*, *S. typhimurium*, *P. vulgaris* and *K. pneumonia* showed reduced susceptibility to BAC across all concentrations when compared to peracetic acid and triameen. Albeit, notably *E. coli* species and *C. jejuni* showed greater levels of susceptibility to BAC, with concentrations of 0.1% producing similar or greater zones of inhibition to that of triameen and peracetic acid. On the other hand, BAC was the most effective disinfectant for *B. subtilis* species and *L. monocytogenes*, providing max zones of 32 mm and 43.5 mm respectively at a concentration of 1%. Notably, a concentration of 0.1% BAC proved to be just as or even more adequate than 1%, as in the case of *B. subtilis* (ATCC) where a zone of 34.5 mm is evident. Albeit peracetic acid and triameen still provided sufficient inhibition levels. For instance, *B. subtilis* (ATCC) was highly susceptible to triameen at 1% (33.5 mm), while at the same concentration peracetic acid produced zones of 31.5 mm, 24 mm and 39 mm for *B. subtilis*, *B. subtilis* (ATCC) and *L. monocytogenes* respectively. Peracetic acid further provided the greatest levels of inhibition for *B. cereus* species, *E. hirae* and *S. aureus* followed by triameen and then BAC, with the exception of MRSA, where BAC provided greater zones of 29.5 mm and 32.5 mm compared to 23.5 mm and 29.5 mm for triameen at concentrations of 0.1% and 1% respectively. In terms of gram-positive test strains, the *B. cereus* species showed the lowest levels of inhibition to all test agents, with an exception seen at 1% peracetic acid, where a significant zone of 39 mm is evident for the isolated strain. Conversely, *E. hirae* and *Staphylococcus* species showed the highest levels of inhibition with zones ranging from 40 mm-47 mm at this same concentration. Of all tested species, *C. jejuni* showed the greatest levels of susceptibility to all disinfectants, with a max zone of 49 mm at 1% peracetic acid, while *K. pneumonia* showed the lowest levels, producing a respective zone of 23 mm. For all test agents under investigation, the presence of BSA at 3 g/L and 10 g/L did influence the diameter of the zone of inhibition **(Table 3)**. For peracetic acid there was an increase in activity for all test strains, though this was not uniform for all concentrations, where specifically *S. typhimurium* showed decreased activity for concentrations of 0.1% and below. The presence of BSA also increased activity of triameen for *E. coli* species, *P. vulgaris* and *E. hirae* species while overall reduced the activity on all other organisms. BAC appeared to be the most hindered chemical in the presence of BSA, where in some instances a significant reduction in zone diameter is evident **(Tables 2 and 3)**. For example, *C. jejuni* and *L. monocytogenes* were particularly affected, showing a 10 mm or greater difference in the presence of 3 g/L BSA, while 10 g/L also decreased the zone diameter for both species. However, this effect was not seen for *P. vulgaris* and *E. hirae* species, where zones either showed an increase or remained relatively the same. Indeed, *P. vulgaris* was the only test species in which BSA continuously provided increased activity of all test chemicals, which was notably uniform at all the concentrations in the presence of 3 g/L, suggesting that the presence of serum albumin somehow increased the cellular uptake of disinfectant by this species.

For isolated species, compared to ATCC reference strains there was no clear pattern where isolated species proved more resistant to QAC exposure than ATCC reference strains, at concentrations below 0.1% however, some variation is evident. For *P. aeruginosa*, the isolated strain appears more susceptible to both triameen and peracetic acid with a decrease in activity seen for isolated *E. hirae* **(Table 2)**. As

such, findings do not contribute any significant data towards the concept of chemical resistance in MDR pathogens.

Table 2: Zones of inhibition (mm) produced test biocidal solutions against MDR pathogenic species associated with foodborne disease as determined by the Kirby Bauer assay (+/- Standard deviation).

			Zones of inhibition (mm)								
		Biocidal solution	BAC			Triameen			Peracetic acid		
		Concentration (%)	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Bacterial test species	Gram negative	*E. coli*	0 (+/-0)	15 (+/-0.3)	20.5 (+/-1.2)	9 (+/-1.1)	16.5 (+/-0.8)	24 (+/-1.1)	10 (+/-1.1)	15 (+/-1.3)	29 (+/-1.7)
		E. coli (ATCC)	4 (+/-1.1)	18.5 (+/-0.6)	21 (+/-1.7)	11.5 (+/-0.7)	17.5 (+/-1.4)	22.5 (+/-0.5)	13 (+/-0.7)	16 (+/-1.1)	27.5 (+/-0.9)
		P. aeruginosa	4 (+/-0.6)	11.5 (+/-1.0)	19.5 (+/-0.8)	20 (+/-1.7)	25 (+/-1.6)	32 (+/-1.7)	22 (+/-0.8)	24.5 (+/-1.7)	39.5 (+/-2.1)
		P. aeruginosa (ATCC)	3.5 (+/-0.6)	12 (+/-0.1)	12 (+/-0.3)	15 (+/-1.5)	18 (+/-1.8)	22.5 (+/-1.1)	25 (+/-0.6)	14.5 (+/-1.2)	32 (+/-1.7)
		S. thymuirium	0 (+/-0)	13.5 (+/-0.1)	14 (+/-1.0)	10.5 (+/-0.8)	20.5 (+/-1.5)	30 (+/-2.1)	13 (+/-0.4)	20 (+/-0.9)	26 (+/-1.2)
		P. vulgaris	0 (+/-0)	10.5 (+/-1.4)	11.5 (+/-1.6)	0 (+/-0)	13.5 (+/-2.3)	17.5 (+/-1.6)	10.5 (+/-1.0)	28 (+/-1.1)	34 (+/-1.2)
		K. pneumonia	0 (+/-0)	14.5 (+/-0.6)	15 (+/-0.8)	12 (+/-1.4)	15 (+/-1.3)	18.5 (+/-1.1)	12.5 (+/-0)	13 (+/-0.3)	23 (+/-0.7)
		C. jejuni (ATCC)	14.5 (+/-1.5)	33 (+/-0.6)	39.5 (+/-1.2)	26 (+/-1.8)	36.5 (+/-2.8)	42 (+/-2.1)	8.5 (+/-0.3)	28.5 (+/-0.8)	49 (+/-1.9)
	Gram positive	*B. subtilis*	20.5 (+/-1.1)	31.5 (+/-1.0)	32 (+/-0.7)	18 (+/-1.1)	23 (+/-1.1)	29 (+/-1.7)	9.5 (+/-0.7)	12 (+/-0.8)	31.5 (+/-1.8)
		B. subtilis (ATCC)	23.5 (+/-1.5)	34.5 (+/-0.5)	32.5 (+/-1.1)	17 (+/-1.8)	27.5 (+/-1.1)	33.5 (+/-0.5)	9 (+/-0.5)	12.5 (+/-0.2)	24 (+/-1.1)
		B. cereus	9.5 (+/-1.1)	20 (+/-1.5)	22 (+/-1.1)	16 (+/-1.7)	20.5 (+/-1.1)	25.5 (+/-0.8)	20.5 (+/-2.2)	24 (+/-1.4)	39 (+/-0.7)
		B. cereus (ATCC)	13.5 (+/-1.4)	29.5 (+/-0.6)	21 (+/-1.9)	16.5 (+/-1.5)	23 (+/-0.1)	25 (+/-0.6)	9.5 (+/-0.9)	13 (+/-1.0)	28.5 (+/-1.2)
		MRSA	17.5 (+/-1.8)	29.5 (+/-1.5)	32.5 (+/-1.2)	18 (+/-0.6)	23.5 (+/-0.9)	29.5 (+/-0.7)	25.5 (+/-1.5)	35.5 (+/-1.8)	46.5 (+/-0.8)
		S. aureus (ATCC)	15.5 (+/-1.3)	29.5 (+/-2.1)	29.5 (+/-1.8)	17 (+/-0.6)	29.5 (+/-1.3)	31.5 (+/-0.8)	26 (+/-0.4)	34 (+/-1.2)	47 (+/-1.5)
		E. hirae	15 (+/-1.1)	25.5 (+/-1.2)	25 (+/-1.1)	16.5 (+/-0.7)	22 (+/-1.5)	28 (+/-1.7)	10 (+/-1.7)	16.5 (+/-0.8)	46.3 (+/-1.2)
		E. hirae (ATCC)	12.4 (+/-0.6)	24.5 (+/-1.7)	28.5 (+/-0.6)	21 (+/-1.1)	26 (+/-1.1)	33 (+/-0.9)	13 (+/-1.0)	20 (+/-1.9)	40 (+/-0.9)
		L. monocytogenes	20.4 (+/-0.9)	41.5 (+/-1/4)	43.5 (+/-0.9)	23.5 (+/-0.8)	28.5 (+/-1.2)	42.5 (+/-1.4)	17.5 (+/-0.9)	17 (+/-1.1)	39 (+/-1.0)

Results of the BS EN 1656 suspension test **(Figure 1)** indicate that all test species were highly susceptible to peracetic acid, triameen and BAC in stipulated high soiling conditions of 10 g/L BSA plus yeast extract. **Figure 1a** reveal high levels of cell death for all test species at a concentration of 0.1%, where a universal 6 $\log_{10}$ reduction in viable cells was achieved within 5 mins for *E. hirae*, *S. aureus*, *P. vulgaris*, and *P aeruginosa* and a 5 $\log_{10}$ death attained for *L. monocytogenes* for all disinfectants. Peracetic acid was the most effective test agent, further reproducing equivalent results at 0.01% for each respective test organism **(Figure 1b)**. While for both triameen and BAC the dose dependent decrease in concentration lead to a 1 $\log_{10}$ decrease in cell death for all test strains, except for *L. monocytogenes*, for which the level of inactivation remained constant. The relevant European testing standards requires a 5 $\log_{10}$ reduction in viable cells within a 30 min period, and thus it should be noted that all three agents met this requirement within 5 minutes at both concentrations (0.01% and 0.1%) for all test species. Further testing against *Bacillus* spores **(Figure 2)** as certain that peracetic acid was the disinfectant of choice,

where **Figure 2** demonstrates a 5 log_{10} reduction of *B. cereus* and *B. subtilis* spores within 45 minutes at a concentration of 2.5%. In fact, peracetic acid met the requirements (10^4 cfu/mL reduction) of this test at 30 mins, where a greater than 4 log_{10} reduction in spores is evident. However, similar results were not attained for triameen, where maximum log_{10} reductions of 3.2 and 1.7 were achieved at 45 mins at a concentration of 5%. Indeed, a dose dependent increase to 7.5% did not improve on its activity (data not shown), and thus triameen failed to meet the relevant European testing standards for this particular test. Spores of *B. cereus* appear more susceptible to triameen at this 5% concentration with similar susceptibility to peracetic acid at 2.5% for both with increasing time.

Table 3: Zones of inhibition (mm) produced test biocidal solutions against ATCC and MDR pathogenic species associated with foodborne disease as determined by the Kirby Bauer assay in the presence of 3 g/L BSA and (10 g/L) BSA inhibitory substance.

			Zones of inhibition (mm)								
		Biocidal solution	BAC			Triameen			Peracetic acid		
		Concentration (%)	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Bacterial test species	Gram negative	*E. coli*	0 (8.5)	15 (16+)	18.5- (17.5-)	16.5 (+/-0.8)	17+ (17.5+)	24.5+ (26+)	13.5+ (15.5+)	17+ (16+)	30+ (31+)
		E. coli (ATCC)	0- (7+)	14- (14.5-)	20- (15.5-)	18+ (11.5)	19+ (19+)	23.5+ (19.5-)	14+ (13.5)	18+ (17.5+)	36.5+ (27+)
		P. aeruginosa	0- (4.5)	11.3 (11.4)	17.5- (17-)	19.5 (20)	22- (22-)	26- (26-)	18.8- (21)	25.5+ (23.3+)	43.5+ (45+)
		P. aeruginosa (ATCC)	4.2+ (4+)	11- (11.5-)	12 (11-)	14.5- (14.5-)	17- (17)-	23.5+ (23.5+)	12.8+ (11.8-)	15.5+ (14.8-)	31.3 (31.5)
		S. thymuirium	0 (8.5+)	5.5- (12.5-)	12- (13-)	14+ (11.5+)	17- (14-)	20- (26.5-)	11- (12-)	19.5- (17-)	33.5+ (17.5-)
		P. vulgaris	0 (0)	11.5+ (10.2)	12+ (10.5)	13+ (12.8+)	15.3+ (14+)	20.4+ (20.5+)	13+ (10)	20.3+ (19+)	39.8+ (40.3+)
		K. pneumonia	0 (0)	14 (13-)	14.7 (13.5-)	13+ (12.5)	14.3- (13.3-)	17- (15.5-)	12.5 (12.3)	14+ (15+)	25.5+ (25+)
		C. jejuni (ATCC)	17+ (13.5-)	21.5- (31-)	25.5- (29.5-)	26 (22-)	29.5- (26-)	42 (33.5-)	16.5+ (15+)	25.5- (18.5-)	50 (33.5-)
	Gram positive	*B. subtilis*	14- (17-)	21- (26-)	22- (29.5-)	17.5- (16.5-)	18.5- (21.5-)	24- (31+)	11+ (7-)	14+ (13+)	28.5- (28.5-)
		B. subtilis (ATCC)	16.5- (18-)	20- (29-)	26- (34-)	19+ (23+)	21- (24-)	27- (33.5+)	8.7 (14+)	12.5 (13)	27+ (29.5+)
		B. cereus	0- (3.5-)	14.5- (17.5-)	18.5- (20-)	15- (11-)	17.5- (19-)	22.5- (21.5-)	23+ (11.5-)	27+ (19-)	41+ (22-)
		B. cereus (ATCC)	4- (11-)	17- (20.5-)	23.2+ (32.5+)	13.5- (14-)	19- (18.5-)	22- (25)	8.5- (10)	15.5+ (12-)	28.5 (24.5-)
		MRSA	15- (14.5-)	23.5- (25-)	26.5- (35.5+)	17.8 (20+)	22- (23)	28- (26.5-)	29.5+ (27.5+)	33.5- (32.8-)	45.5- (44-)
		S. aureus (ATCC)	14- (16.5+)	22.5- (29.5)	26- (30-)	18.5+ (20+)	24- (25.3-)	25.5- (31)	26.5 (27.5+)	33- (30-)	49.5+ (44-)
		E. hirae	15 (15.5)	26.5+ (25.5)	25 (25)	18+ (17.5+)	23+ (23.4+)	26.5- (25-)	10 (10.5)	20+ (15-)	45- (43.5-)
		E. hirae (ATCC)	15.8+ (17.7+)	26.8+ (27+)	28.8 (26.3-)	23+ (25.2+)	30+ (30+)	33 (37.5+)	13 (11.5-)	23.3+ (24.8+)	45.5+ (41+)
		L. monocytogenes	19- (19-)	32.5- (29-)	32.5- (33-)	21- (21-)	30+ (31+)	33.5- (39-)	18.5+ (17)	19.5+ (20+)	45+ (40+)

+ indicates increase zone diameter compared to absence of BSA; - indicates decrease zone diameter compared to absence of BSA

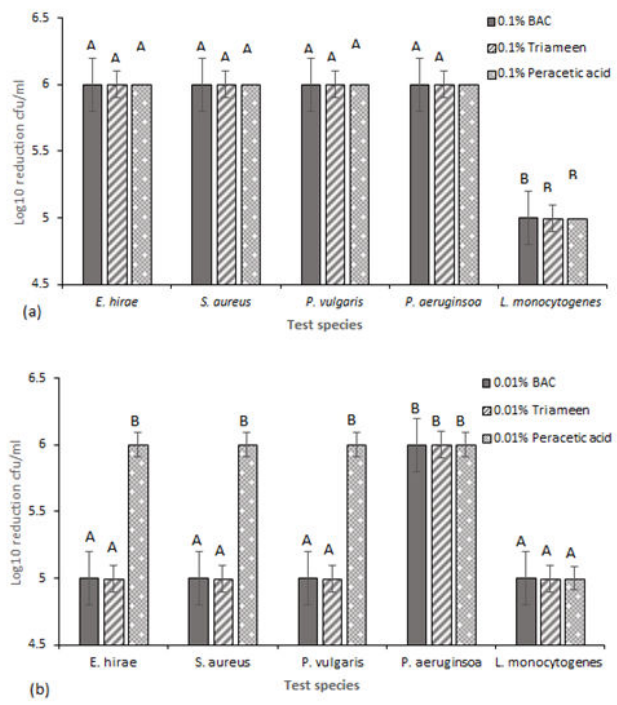

Figure 1: Log_{10} reduction in viable cfu/ml of test species as determined by BSEN 1656 following 5 minutes exposure to (a) 0.1% and (b) 0.01% BAC, triameen and peracetic acid in high interfering solution 10 g/L BSA and yeast extract (+/- Standard deviation). A and B indicate significant difference at p>0.05.

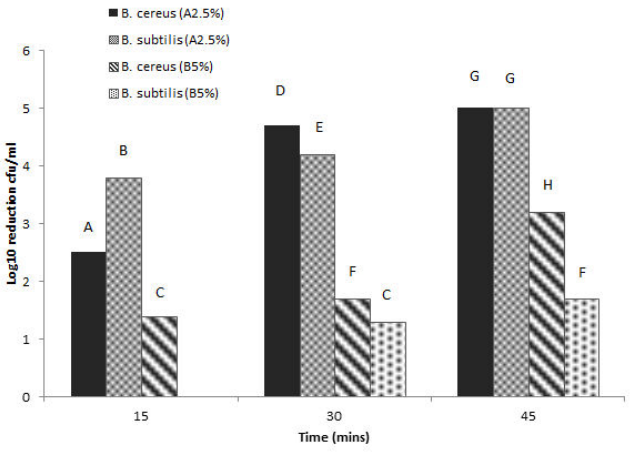

Figure 2: Log_{10} reduction of pathogenic MDR isolates of *B. cereus* and *B. subtilis* endospores following exposure to (A) 2.5% peracetic acid and (B) 5% triameen test solution in accordance with the BSEN 13704 (+/-S.D). A, B, C, D, E, F, G and H indicate significant difference at p>0.05.

Discussion

Foodborne disease is a global issue with WHO estimates of 600 million cases and 420,000 deaths annually worldwide, with children under 5 particularly affected [10]. Bacterial species frequently associated with foodborne disease include serovars of *E. coli*, *Salmonella*, *C. jejuni*, *Pseudomonas*, *Bacillus*, *Staphylococcus* and *L. monocytogenes* [1]. Where the food related bacterial pathogens *Pseudomonas*, *Salmonella*, *E. coli*, *Staphylococcus* and *Campylobacter* are all listed on the WHO priority pathogen list. In a time of antibiotic and multidrug resistance the importance of disease prevention cannot be overlooked. An adequate, timely, and effective disinfection protocol remains one of the most important means of preventing transmission of these microbial species. In food production and processing environments sanitizing animal housing areas, slaughter equipment, food utensils, contact surfaces and additional fomites is essential to prevent food contamination. Furthermore, microbial biofilms on contact surfaces are typically more MDR and chemical resistant than their planktonic counterparts. Interestingly, the contamination of carcasses at slaughter has been correlated to the prevalence of *E. coli* O157:H7 in cattle faeces [3]. Food contamination with microbial pathogens is typically a result of contaminated raw material, ineffective processing techniques including thermal processing and contamination post treatment. Studies show that an efficient cleaning procedure can reduce the bacterial load on food processing equipment by up to 99.8% [11]. Quaternary ammonium compounds are one of most universally used disinfectants in the food sector due to their potent antimicrobial activity. Issues arose however, relating to QAC residues detected in a broad range of food items exceeding the default Maximum Residual Level (MRL) of 0.5 mg/kg set out by the EU. This guideline was subsequently replaced with an MRL of 0.1 mg/kg by the EU commission in 2014, consequently reducing the safe application of QACs in food industries. Studies also report the cross resistance between MDR pathogens and QAC compounds such as BAC and DDAC, further raising concerns with their continued application. Environmental pollution with these compounds is an issue as they are toxic to aquatic organisms including fish, daphnids, algae, rotifer, and microorganisms active in wastewater treatment systems [12]. To circumvent these issues novel biocidal options, need to be investigated. Studies described herein describe the efficacy of peracetic acid and triameen in comparison to BAC for the biocidal inactivation of foodborne bacterial species. Both disinfectants previously proved effective at inactivating similar isolates relevant to both clinical [13] and bovine mastitis isolates [14]. MDR species investigated display high susceptibility to both biocides with peracetic acid providing satisfactory level of Bacillus spore inactivation. Indeed, peracetic acid proves a more efficient biocide than BAC in all studies in the absence and presence of interfering substance. Peracetic acid is a potent biocidal oxidizer that retains its efficacy in the presence of organic matter. As such it is used in the medical sector for the sterilization of medical and surgical equipment at a concentration of 0.2% (2000 ppm). Studies described here also demonstrate the efficacy of peracetic acid against numerous

MDR bacterial species. In terms of susceptibility variations between Gram-negative and Gram-positive species, no clear pattern emerged where one group appear more susceptible than the other for any biocide investigated. In the Gram-negative cohort of isolated species, the order of susceptibility (highest to lowest) to peracetic acid is *P. vulgaris*, *P. aeruginosa*, *S. thymuirium*, *E. coli* and *K. pneumonia*. For triameen the order is as follows *P. aeruginosa*, *S. thymuirium*, *E. coli*, *K. pneumonia* and *P. vulgaris*. Both biocides provided significantly higher levels of inactivation for all Gram-negative species than BAC. Interesting, the most antibiotic resistance strain *P. aeruginosa* was one of the most susceptible species investigated. *C. jejuni* was clearly the most susceptible species to both biocides, as the ATCC reference strains do not show a definite pattern of increasing or decreasing resistance comparative to their MDR isolated counterpart, the high levels of inactivation of this strain are promising. For all test biocides however, the level of susceptibility was reduced in the presence of interfering organic matter for *C. jejuni*. *C. jejuni* is commonly associated with foodborne disease and represents a significant issue for the poultry industry as an enteric species of bird intestinal tracts. At present, *C. jejuni* and *C. coli* are the leading causes of enteric infections in many developed countries [15] where incidence of Guillain-Barré syndrome have been associated with infection. Further difficulty arises with this species as under adverse growth conditions, *Campylobacter* can form viable but non-culturable cells making viability difficult to determine. *E. coli* was also highly inactivated by both biocides, further studies assessing shiga toxin-producing *E. coli* (STEC), Verocytotoxigenic *E. coli* (VTEC) and 0157 are also warranted. Poultry and bovine meat have been shown to frequently contain *E. coli* serovars. In the isolated Gram-positive cohort, the order of susceptibility from highest to lowest to peracetic acid is *MRSA*, *E. hirae*, *B. cereus*, *L. monocytogenes* and *B. subtilis*. For triameen findings show an order of (from highest to lowest susceptibility) *L. monocytogenes*, *MRSA*, *B. subtilis*, *E. hirae* and *B. cereus*. Interestingly, *L. monocytogenes* appears more susceptible to triameen suggesting in food preparation areas of high risk for *Listeria* contamination this could be a suitable biocidal option, as it also appears more effective than peracetic acid for this strain. Although, unlike with peracetic acid this activity was somewhat inhibited in the presence of interfering organic matter. *Listeria* is a soil organism associated with non-pasteurized milk, milk products, and vegetables. Infection results in varying levels of disease from febrile gastroenteritis in immunocompetent persons to bacteraemia, meningitis, fatality (30%) in immunocompromised persons and spontaneous abortions in pregnant patients [16]. Food preparation surfaces in dairy and vegetable production and clinical settings (housing immunocompromised persons) may be best disinfected with between 0.1% and 1% triameen to reduce viable *Listeria* numbers. All 3 test chemicals satisfied the requirements of BSEN 1656 providing a 5 $\log_{10}$ inactivation of required strains and *L. monocytogenes* within 5 mins exposure. *L. monocytogenes* does appear more resistant than all other test strains however, with 1 $\log_{10}$ less cell death for all chemicals up to 0.1%. The MDR isolate of *MRSA* (resistant to Erythromycin, Streptomycin, Ampicillin and intrinsically resistant to Cefpodoxime) appears more susceptible to peracetic acid than BAC and triameen. *MRSA* is recognised as an important hospital acquired, zoonotic and foodborne (poultry, pork, beef, milk and vegetables) pathogen where studies have detected the heat labile *S. aureus* enterotoxin in several foodborne *MRSA* isolates [17]. BSEN 1656 is a suspension test detailing specification to be met by disinfectants for use in the veterinary area. All testing species are zoonotic pathogens gaining entry to food primarily pre-harvest and at harvest making this a relevant test to control these environmental pathogens. Additionally, previous studies report the activity of peracetic acid and triameen as determined by BSEN 1276 (for use in domestic, industrial and food areas) against numerous strains [13]. Peracetic acid displays greater sporicidal activity than triameen meeting the requirements of BSEN 13704 within 30 minutes for both *B. cereus* and *B. subtilis* at 2.5% (2500 ppm). QAC compounds are not sporicidal but are sporostatic, at concentrations of 5 ppm [18]. The sporicidal activity of peracetic acid is of importance as these thermoduric robust cells are resistance to many food processing techniques including pasteurization, refrigeration, radiation, and chemical exposure [19]. *B. cereus* represents a major concern to food producers as it produces 2 types of toxins, an emetic toxin (heat-stable cerulide) and diarrheal enterotoxins associated with food poisoning in its vegetative state.

Conclusion

Foodborne disease is major issue causing incidence of morbidity, mortality, and economic losses globally. Often self-limiting in nature, reported cases of disease are often greatly underestimated. Extra intestinal infections resultant from foodborne disease is also not reported in pathogens which have surveillance systems. Prevention is the best approach to deal with drug resistant microbial disease. As the prevalence of resistant infection and mortality increases globally, the importance of disease prevention is undeniable. The food sector represents a major source of disease transmission of numerous pathogens which may induce varying levels of pathogenicity. The presence of antibiotic resistant and multi drug resistant strains such as *MRSA*, *Salmonella*, *Klebsiella*, *Pseudomonas* and *E. coli* species (amongst others) causes difficulties in treating illness relating to these strains. Furthermore, the presence of resistance genes in the environment results in the proliferation of resistance in foodborne and environmental pathogens. In immunocompromised individuals, neonates and elderly any foodborne pathogen may cause severe illness and morbidity. The resistance of many of these species to food processing techniques is already evident due to their varying virulence factors. Findings of this study show that both triameen and peracetic have high levels of antibacterial activity with peracetic acid also demonstrating sporicidal activity. Both biocides could be implemented within food processing, animal housing and slaughter environments to control the spread of microbial species. Such prevention measures ensuring animal health are a key aspect of the One Health approach protecting the food chain by protecting food producing animals.

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