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REVIEW ARTICLE

Impact of benzalkonium chloride, benzethonium chloride and chloroxylenol on bacterial antimicrobial resistance

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Abstract

This review examined 3655 articles on benzalkonium chloride (BKC), benzethonium chloride (BZT) and chloroxylenol (CHO) aiming to understand their impact on antimicrobial resistance. Following the application of inclusion/exclusion criteria, only 230 articles were retained for analysis; 212 concerned BKC, with only 18 for CHO and BZT. Seventy-eight percent of studies used MIC to measure BKC efficacy. Very few studies defined the term 'resistance' and 85% of studies defined 'resistance' as <10-fold increase (40% as low as 2-fold) in MIC. Only a few in vitro studies reported on formulated products and when they did, products performed better. In vitro studies looking at the impact of BKC exposure on bacterial resistance used either a stepwise training protocol or exposure to constant BKC concentrations. In these, BKC exposure resulted in elevated MIC or/and MBC, often associated with efflux, and at time, a change in antibiotic susceptibility profile. The clinical relevance of these findings was, however, neither reported nor addressed. Of note, several studies reported that bacterial strains with an elevated MIC or MBC remained susceptible to the in-use BKC concentration. BKC exposure was shown to reduce bacterial diversity in complex microbial microcosms, although the clinical significance of such a change has not been established. The impact of BKC exposure on the dissemination of resistant genes (notably efflux) remains speculative, although it manifests that clinical, veterinary and food isolates with elevated BKC MIC carried multiple efflux pump genes. The correlation between BKC usage and gene carriage, maintenance and dissemination has also not been established. The lack of clinical interpretation and significance in these studies does not allow to establish with certainty the role of BKC on AMR in practice. The limited literature and BZT and CHO do not allow to conclude that these will impact negatively on emerging bacterial resistance in practice.

KEYWORDS

benzalkonium chloride, benzethonium chloride, chloroxylenol, cross-resistance, resistance

INTRODUCTION

There are many terms used in the literature to describe biocide 'resistance', including tolerance, decreased susceptibility, reduced susceptibility, insusceptibility, multidrug resistance, intrinsic or innate resistance, acquired resistance, acquired reduced susceptibility, co-resistance and cross-resistance (Gerba & Müller, 2015; Maillard et al., 2013; Meade et al., 2021). Some of these terms clearly reflect the lack of consensus within the scientific community. It also adds to the difficulty in comparing studies where the term 'resistance' is loosely used. In this review, the use of hyphenated 'resistance' reflects the terminology used in the source article, whilst *resistance* refers to bacterial survival in a biocidal product or bacterial survival at an in-use concentration of a biocide in a product. *Decreased susceptibility* refers to an upward change in MIC or MBC of a biocide. *Tolerance* refers only to bacterial growth in the presence of a low (MIC range) concentration of a biocide (Gerba & Müller, 2015).

Bacterial decreased susceptibility and resistance to biocides has been reported since the 1950s, notably with quaternary ammonium compounds (Maillard, 2018; Maillard et al., 2013). To date, bacterial decreased susceptibility and resistance has been described with all biocides, including highly reactive ones such as alkylating and oxidizing agents (Maillard, 2018; Maillard et al., 2013). Biocides have general multiple and non-specific target sites against bacteria, which suggests that bacterial resistance might be difficult to emerge when a biocide is used at a high concentration, generally above the minimum bactericidal concentration; this holds mostly true (Gerba & Müller, 2015; Maillard, 2007; Maillard, 2018; Meade et al., 2021).

The clinical or industrial implications of bacteria with a reduced biocide susceptibility are unclear at present. Bacteria surviving in a biocidal product have led to documented bacterial infections in patients, although misuse of the product or the presence of intrinsically resistant bacterial spores was the reason for the presence of bacterial pathogens (Weber et al., 2007). Bacterial decreased susceptibility to cationic agents such as biguanides and quaternary ammonium compounds, and phenolics such as triclosan has been widely reported in vitro and is often perceived to present a higher risk for the development of bacterial resistance to antimicrobials compared to highly reactive biocides such as oxidizing- and alkylating-based biocides. This perceived increased risk is related to product mechanism of action, product usage or persistence in the environment (http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf, accessed 16/11/21). Evidence of decreased susceptibility to biocides mainly comes from in vitro investigations and is often defined as an increase in the minimum inhibitory concentrations (MIC), regardless of the in-use concentration of the biocide, the composition of the formulation or its application (Maillard, 2018; Maillard et al., 2013).

Since 2016, the US Food and Drug Administration (US-FDA) issued several rules to determine the safety and effectiveness of consumer antibacterial-based products, particularly those containing benzalkonium chloride (BKC), benzethonium chloride (BZT) and chloroxylenol (CHO) (*Safety and Effectiveness of Consumer Antiseptics: Topical Antimicrobial Drug Products for Over-the-Counter Human Use*, *Safety and Effectiveness of Health Care Antiseptics: Topical Antimicrobial Drug Products for Over-the-Counter Human Use* and *Safety* and *Effectiveness of Consumer Antiseptic Rubs: Topical Antimicrobial Drug Products for Over-the-Counter Human Use*). These rules are based partly on the concern that long-term exposure to certain active ingredients, such as triclosan, may be associated with bacterial resistance and thus pose a health risk.

This review aims to provide an in-depth analysis of the peer-reviewed literature regarding the potential for the topical antimicrobial active ingredients benzalkonium chloride, benzethonium chloride and chloroxylenol to confer antimicrobial 'resistance' in bacteria. This review assessed, compiled and critically appraised the relevant literature available from scientific databases regarding the impact of bacterial exposure to these three biocides on antimicrobial 'resistance'.

SELECTION OF RELEVANT LITERATURE

The databases searched were *Scopus, PubMed*, *Web of Science* and *Google Scholar*. In addition, a private database, comprising co-prising of scientific articles, reviews and industrial reports (provided by the American Cleaning Institute) was used. A total of 3655 scientific articles that mentioned BKC, BZT and CHO were analysed. After duplicate outputs were removed and the exclusion/inclusion criteria applied, only 230 were retained for an in-depth analysis. The literature was analysed up to 25 January 2022.

Exclusion and inclusion criteria

This review concerns vegetative bacteria of interest, particularly those mentioned in the US-FDA *Safety and Effectiveness of Consumer Antiseptic Rubs; Topical Antimicrobial Drug Products for Over-the-Counter Human Use* (https://www.federalregister.gov/documents/2019/04/12/2019-06791/safety-and-effectiveness-of-consumer-antiseptic-rubs-topical-antimicrob

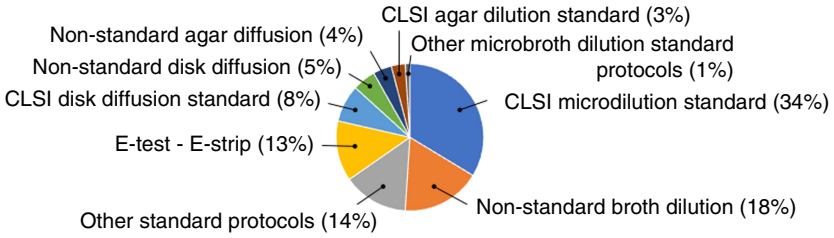

FIGURE 1 Protocols used for the determination of antibiotic susceptibility of bacterial isolates. (based on 98 scientific articles).

ial-drug-products-for; accessed 19/04/2022). Biocide efficacy against bacterial biofilm was not considered, but the impact of biocide exposure on biofilm formation and composition was. Bacterial (endo)spores are intrinsically resistant to BKC, BZT and CHO and were excluded. Only peer-reviewed scientific articles written in English were considered and not peer-reviewed review articles.

To focus the review on relevant literature effectively, initially, several keywords were used including commercial and chemical names for benzalkonium chloride, benzethonium chloride and chloroxylenol, but also 'quaternary ammonium compounds' or QAC, and phenolics. Then, exclusion criteria included lack of mentioning BKC, BZT and CHO, lack of detailed MIC/MBC (minimum bactericidal concentration) determination protocols or other protocols to determine bactericidal efficacy, lack of a detailed antibiotic susceptibility determination protocol, lack of mentioning 'resistance' and non-relevant microorganisms such as fungi, protozoa and spores.

Typically keywords and exclusion criteria to retain eligible documents followed a screen from (i) abstract level and (ii) full-text level. Each retained article was then assessed for its relevance to this review in terms of bacterial susceptibility or resistance to the three actives. Finally, a detailed analysis of the test protocol used (MIC/MBC determination, antibiotic susceptibility assay), expression of resistance genes, evidence of stress response, mechanisms of resistance, maintenance of resistance genes and virulence assay, was performed for all the selected articles.

'Resistance' and definitions

Overall, only a minority of studies defined the term 'resistance'. The majority of studies used the term 'resistance' to describe an increase in MIC between 2- and 10-folds (87%) and as low as 2-fold (40 %). Some studies defined 'resistance' as an MIC for an isolate above the MIC for 90% or 99% of bacteria of the same species. In this review, the hyphenated term 'resistance' was used to reflect the terminology reported in these studies. Where appropriate the use of decreased susceptibility or tolerance were used as defined in the introduction. Very few studies investigated formulations or *in-use* or *during use* concentrations reflecting product usage in practice (see below). The *during use* concentration reflects potential dilution of the product upon usage, whilst the *in-use* concentration is the concentration to use recommended by the manufacturer (Wesgate et al., 2016). Where bacteria survived, *the in use* or *during use* concentration of a product the term resistance was used.

When chemotherapeutic antibiotics were concerned, the term 'resistance' means clinical resistance and refers to breakpoints given by antimicrobial test standards. Yet, 27% of studies did not use recognized antibiotic standard testing protocols, whilst the majority do not refer to associated clinical breakpoints (Figure 1), but instead defined 'resistance' as a decrease in susceptibility, recorded as zone of inhibition or MIC, with no clinical significance. Where clinical significance (or lack of) is mentioned, this referred to clinical breakpoint given for a specific antibiotic from an established organization, such as for example, the British Society for Antimicrobial Chemotherapy (Andrews, 2009), the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2014) or the International Standard Organisation (ISO, 2006).

BENZALKONIUM CHLORIDE AND BACTERIAL 'RESISTANCE'

Benzalkonium chloride (CAS: 8001-54-5) is a cationic biocide with a broad-spectrum activity except for bacterial endospores (Leggett et al., 2016). Its bactericidal efficacy has been associated with an alteration of cytoplasmic membrane permeability following dissociation of cellular membrane lipid bilayers, resulting in leakage of cellular contents (Knauf et al., 2018).

Two hundred and twelve peer-reviewed scientific papers investigating bacterial susceptibility, decreased susceptibility or 'resistance' to benzalkonium chloride (BKC) were retained and analysed (Table S1). The majority of the papers were classified as food-related (31%), clinically related (29%) and laboratory-related (25%) studies (Figure 2). The laboratory-related studies concern the use of bacterial strains from culture collection, laboratory-derived mutants or genetic constructs to investigate specific mechanisms of resistance, for the majority (80%), efflux pumps (Figure 3). The majority of articles investigated specific pathogens, such as *staphylococci* (mostly *Staphylococcus*

aureus), *Listeria monocytogenes*, pseudomonads (mostly *Pseudomonas aeruginosa*), *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumonia* and *Acinetobacter baumannii*. A few studies concerned enterococci, *Burkholderia* spp., *Stenotrophomonas* spp., *Serratia marcescens*, *Enterobacter* spp. and *Campylobacter* spp. (Table S1).

Susceptibility determination protocols

Most studies measured a change in MIC or MBC based on standard efficacy protocols, particularly the Clinical and Laboratory Standards Institute (CLSI) microdilution broth protocol (Figure 4). For most studies (Table S1), 'resistance' is based on a change in MIC as low as 2-fold increase (40%) and 10-fold increase (85%) (Figure 5). A < 10-fold increase in MIC observed could be considered as marginal (Maillard et al., 2013; Russell & McDonnell, 2000). A more substantial increase (>10-fold) in MIC was only reported in six studies (Braoudaki & Hilton, 2005; Chaplin, 1951; Correa et al., 2008; Guo et al., 2015; Joynson et al., 2002; Loughlin et al., 2002). The CLSI microbroth dilution protocol was the most frequently used (34% of studies) to determine changes in antibiotic susceptibility profiles (Figure 1).

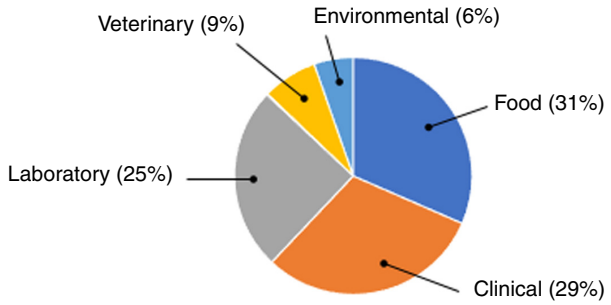

FIGURE 2 Main areas covered from peer-reviewed scientific articles on BKC. *Food* includes all food pathogens; *Clinical* includes all human pathogens; *Laboratory* refers to the use of constructs regardless of the bacterium studied; *Veterinary* includes animal pathogens; *Environmental* including environmental and plant bacteria. (based on 212 scientific articles).

Impact of bacterial exposure to BKC

Bacterial exposure to low (sub-MIC) concentrations of BKC was investigated in several studies, the majority of which related to food and laboratory environments. These in vitro studies generally aimed to understand the mechanisms driving a reduction in antimicrobial susceptibility in adapted strains.

Thirty-five peer-reviewed articles (17% of retained BKC studies) investigated the impact of BKC exposure on increase in BKC MIC (Adair et al., 1975; Braoudaki & Hilton, 2004, 2005; Capita et al., 2019; Chaplin, 1951; Chuanchuen et al., 2008; Condell et al., 2012; Curiao et al., 2015; Dejoies et al., 2021; Fernández-Cuenca et al., 2015; Guérin et al., 2021; Joynson et al., 2002; Kawamura-Sato et al., 2008; Kim et al., 2018; Knapp et al., 2013, 2015; Knauf et al., 2017; Langsrud et al., 2004; Ligowska-Marzeta et al., 2019; Loughlin et al., 2002; Marzoli et al., 2021; Mavri & Smole Možina, 2013; Nhung et al., 2015; Noll et al., 2020; Pagedar et al., 2012; Puangseree et al., 2021; Rakic-Martinez et al., 2011; Romeu et al., 2020; Sánchez et al., 2015; Soumet et al., 2012; Tabata et al., 2003; Tandukar et al., 2013; To et al., 2002; Voumard et al., 2020; Zhang et al., 2012). Nineteen of these used an artificial stepwise training protocol that relies on increasing BKC concentrations in a stepwise fashion (Table 1).

A high BKC MIC post stepwise training (100,000 mg l^{-1}) was reported in *S. marcescens* in only one early study (Chaplin, 1951) and such results have not been reported since albeit with other bacteria. Other studies used single or continuous exposure to the same BKC concentration over different time periods and reported a more modest increase in BKC MIC (2–4-fold increase) (Table 1). Only one study reported 100-fold increase in MIC *S. enterica* serovar Typhimurium following exposure to during use concentration - 0.015 or 0.004 mg l^{-1} (Knapp et al., 2015). Overall, the highest MIC (460 mg l^{-1}) was reported in a complex microcosm (Tandukar et al., 2013).

Based on the retained literature on BKC, 54% (114/212) of the studies investigated the antibiotic susceptibility profile of isolates. Of those, 73% (83/114) used accepted

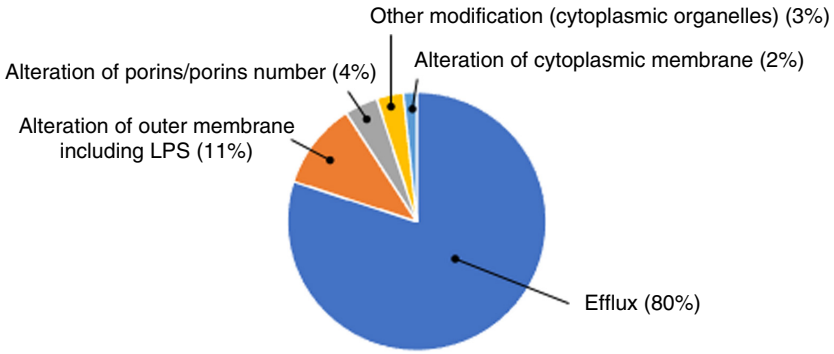

FIGURE 3 Mechanisms associated with a decrease in BKC susceptibility or/and a change in antibiotic susceptibility profile. Efflux includes direct or indirect measurements, using efflux inhibitors or/and fluorescent substrates, or gene expression. (based on 120 scientific articles).

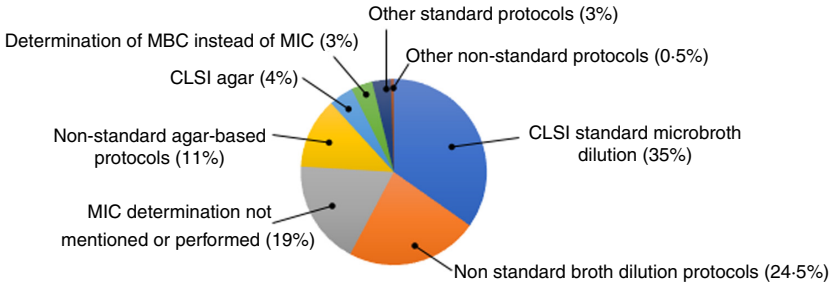

FIGURE 4 Protocols used for the determination of antimicrobial activity of BKC. (based on 212 scientific articles).

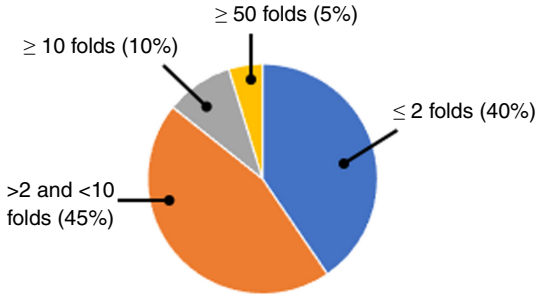

FIGURE 5 Percentage of studies assessing BKC 'resistance' based on increased folds in MIC. (based on 45 scientific articles).

standardized antibiotic protocols such as those recommended by CLSI, BSAC or the use of e-test (Figure 1). Most of these studies investigated the antibiotic susceptibility profile of clinical and environmental isolates (Table S1). Bacteria adapted to increasing concentrations of BKC or following BKC single exposure were often found to have a reduced susceptibility to antibiotics in mostly Gram-negative bacteria such as *P. aeruginosa* (Adair et al., 1975; Joynson et al., 2002; Kim et al., 2018; Loughlin et al., 2002; Morita et al., 2003; Pagedar et al., 2011; Voumard et al., 2020), *E. coli* (Curiao et al., 2015; Forbes et al., 2016; Langsrud et al., 2004; Nhung et al., 2015; Pagedar et al., 2012; Pereira et al., 2021; Puangseree et al., 2021), *Burkholderia* spp. (Geftic et al., 1979; Knapp et al., 2013, 2015), *Salmonella* spp. (Braoudaki & Hilton, 2004; Chuanchuen et al., 2008; Condell et al., 2012; Nhung et al., 2015), *Klebsiella pneumoniae* (Curiao et al., 2015), *A. baumannii* (Morrissey et al., 2004), *L. monocytogenes* (Guérin et al., 2021; Pasquali et al., 2018; Rakic-Martinez et al., 2011), *Campylobacter* spp. (Mavri & Smole Možina, 2013), but also against staphylococci (Forbes et al., 2016; Houari & Di Martino, 2007; Marzoli et al., 2021), or against diverse isolates from food sources (Gadea et al., 2017) or water effluents (Tandukar et al., 2013). Only three studies reported a clinical change in antibiotic susceptibility (Knapp et al., 2013; Pereira et al., 2021; Puangseree et al., 2021). Out of 16 studies investigating the effect of BKC pre-exposure on changes in antibiotic profile, only four investigated the persistence of this change (Guérin et al., 2021; Knapp et al., 2013; Mavri & Smole Možina, 2013; Voumard et al., 2020). Increase in antibiotic MIC (Mavri & Smole Možina, 2013), change in antibiotic susceptibility (Guérin et al., 2021) or clinical resistance (Knapp et al., 2013) was found to be unstable in the absence of BKC. The remaining studies did not investigate the stability of susceptibility profile change and did not consider the clinical significance of the observed changes (Table S1).

Capita et al. (2019) reported a change in ciprofloxacin clinical susceptibility from sensitive to intermediate in *Cronobacter sakazakii*. Other studies observed no change in antibiotic susceptibility profile (Braoudaki & Hilton, 2004; Furi et al., 2013; Knapp et al., 2015) or fitness cost (Curiao et al., 2015; Furi et al., 2013; Sánchez et al., 2015; Soumet et al., 2012) after BKC exposure. One recent study observed that *E. coli* mutants (with altered porins and efflux pumps) which evolved for 500 generations in BKC 4 $mg l^{-1}$, showed higher fitness in competition assay with the wild strain (Pereira et al., 2021). Maertens et al. (2020) observed that *E. coli* isolates pre-exposure to 6.75 $mg l^{-1}$ BKC decreased the bactericidal activity of ciprofloxacin, presumably following a decrease in membrane potential inducing dormancy in some bacteria within the cell population. Such observation has not been confirmed in situ. Formation of viable but non-culturable cells (VBNC) following BKC exposure (0.008 $mg l^{-1}$ at 37°C for 24 h) has also recently been reported in *L. monocytogenes*. Following stepwise training, adapted bacteria showed a 2-fold increase in BKC MIC and decreased antibiotic susceptibility to ceftriaxone, gentamicin, linezolid, tetracycline and trimethoprim/sulphamethoxazole. These observations likely resulted from an alteration in *L. monocytogenes* membrane permeability (Noll et al., 2020). Knapp et al. (2013) reported clinical susceptibility changes for ceftazidime, imipenem and ciprofloxacin in *Burkholderia lata* following exposure to BKC. These changes were however random and were only present in 50% of the experimental repeats. Adaptation of bacteria to BKC in terms of increased MIC has been found to be stable in *S. enterica* (Braoudaki & Hilton, 2004, 2005; Chuanchuen et al., 2008) and in *P. aeruginosa* (Voumard et al., 2020), but transient in *S. aureus* (Buzón-Durán et al., 2017) and *B. lata*. (Knapp et al., 2013). A recent study based on *P. aeruginosa* pre-exposure to BKC (40–70 $mg l^{-1}$; corresponding to 50% or 88% MIC) showed no change in clinical susceptibility to antibiotics of the isolates despite

TABLE 1 Impact of BKC exposure/treatment on antimicrobial susceptibility in bacteria

Bacteria	Exposure/treatment	MIC before exposure	MIC post exposure	Reference
Stepwise training				
P. aeruginosa ATCC 9027	Subculture in increasing concentrations of BKC	ND	MIC: >200 mg l^{-1}	Adair et al. (1975)
E. coli O157; *S enterica* serovar enteritidis, typhimurium, virchow	Growth in increasing concentration - starting sub-MIC level	MIC: 400 mg l^{-1} *S. enterica*, virchow and enterica, <100 mg l^{-1} virchow	MIC: >1000 mg l^{-1} for *E. coli*	Braoudaki and Hilton (2004)
S enterica serovar enteritidis, typhimurium, virchow	Growth in increasing concentration - starting sub-MIC level	MIC for *S. enterica* serovar enteritidis 32 mg l^{-1}, virchow 256 mg l^{-1} and typhimurium 64 mg l^{-1}	MIC for *S. enterica* serovar enteritidis 256 mg l^{-1}; virchow 256 mg l^{-1} and typhimurium from 64 mg l^{-1}	Braoudaki and Hilton (2005)
Cronobacter sakazakii and enterocolitica	Subculture in increasing concentrations of BKC; starting sub-MIC level	MIC: 15 mg l^{-1} for *C. sakazakii* MIC: 20 mg l^{-1} for *Y. enterocolitica*	MIC: 56.95 mg l^{-1} for *C. sakazakii* MIC: 50.63 mg l^{-1} for *Y. enterocolitica*	Capita et al. (2019)
S. marcescens (Breed's no. 1377)	Subculture in increasing concentrations of BKC from highest concentration showing growth	MIC: 50–100 mg l^{-1}	MIC: 100,000 mg l^{-1} in three passages over a period of 15 days, and 50,000 mg l^{-1} in eight passages during 45 days	Chaplin (1951)
S. enterica isolates from poultry ($n = 125$) and swine ($n = 132$)	Exposure to sub-inhibitory concentrations	MIC: 32–256 mg l^{-1} - most at 64 mg l^{-1}	MIC: 128–256 mg l^{-1}	Chuanchuen et al. (2008)
189 *Salmonella* strains, including 48 serotypes from various origins (such as clinical sources, food, the environment and water)	Growth in (0.25 or 0.5 MIC). Biocide formulation concentration increased in a stepwise manner until the biocide concentration reached 4X MIC	MIC:15 mg l^{-1}	MIC: 50 mg l^{-1}	Condell et al. (2014)
E. coli and *K. pneumoniae* mutants with different TRI susceptibility	Growth in sub-inhibitory concentrations (0.5 MIC), followed by agar with concentration of 2.5 to 33 × MIC	MIC: 16 mg l^{-1}	MIC: 32 mg l^{-1}	Curiao et al. (2015)
P. aeruginosa NCIMB 10421 clinical isolates of *P. aeruginosa*	Subculture in increasing concentrations of BKC (2-fold steps)	MIC: 50 mg l^{-1}	MIC: 580 mg l^{-1} after 25 subcultures	Joynson et al. (2002)
P. aeruginosa	Subculture in increasing concentrations of BKC until cessation of growth	MIC: 200 mg l^{-1}	Growth in BKC up to 1600 mg l^{-1}	Kim et al. (2018)
16 *P. aeruginosa* from hospital environment	Subculture in increasing concentrations of BKC	MIC: 15–30 mg l^{-1}	MIC: >500 mg l^{-1} for clinical isolate; Reference standard culture strain PAO1: 500 mg l^{-1}	Loughlin et al. (2002)
Three *C. jejuni* (NCTC11168, ATCC33560 and the K49/4 poultry isolate) and two *C. coli* (ATCC33559, and the 137 poultry isolate)	Subculture in increasing concentrations of BKC	MIC: 0.5–2 mg l^{-1}	1–4-fold increase in MIC; ATCC33559 strain: 4-fold MIC increase after 15 days of exposure	Mavri and Smole Možina (2000)

(Continues)

TABLE 1 (Continued)

Bacteria	Exposure/treatment	MIC before exposure	MIC post exposure	Reference
6 antimicrobial-susceptible *E. coli* and 6 antimicrobial-susceptible non-typhoidal *Salmonella* (NTS) isolates	Subculture in increasing concentrations of BKC: 0.5 MIC exposure over 12 days	MIC: 21–30 mg l^{-1} for *Salmonella* and 12–27 mg l^{-1} for *E. coli*	Post exposure MIC increase: <2-fold to 2-fold in one *Salmonella* and one *E. coli*	Nhung et al. (2015)
L. monocytogenes SLCC2540	Subculture in increasing concentrations of BKC; starting concentration of 0.002 mg l^{-1}; growth in increasing concentration of 0.001 mg l^{-1} every 48 h	MICL: 0.004 mg l^{-1} MBC: 0.011 mg l^{-1}	MIC: 0.008 mg l^{-1} MBC: unchanged	Noll et al. (2020)
81 *E. coli* from biofilm collected from raw milk line, pasteurizer inlet, outlet and, milk tank of a small scale and commercial scale dairy	Subculture in increasing concentrations of BKC; starting with sub-MIC (well immediately below MIC step of 10 mg l^{-1})	MIC: 50–250 mg l^{-1}	MIC: 70–350 mg l^{-1}; largest increased from 50 to 130 mg l^{-1}	Pagedar et al. (2012)
E. coli veterinary isolates	Subculture in increasing concentrations of BKC starting 1/4 MIC for 24 h	MIC range from 4 to 64 mg l^{-1}	Only 2/24 isolates showed increased MIC >4-fold (6- and 9-folds increased). All the others <2-fold	Puangseree et al. (2021)
9 avian and porcine *E. coli* strains and *E. coli* ATCC25922	Subculture in increasing concentrations of BKC daily for 7 days	MIC: 16–32 mg l^{-1}	2-fold increase in MIC	Soumet et al. (2012)
P. aeruginosa ATCC 10145 and deletion mutants	Subculture in increasing concentrations of BKC	MIC: 18.2 mg l^{-1}	MIC: 51.2 mg l^{-1}	Tabata et al. (2003)
Two resistant (4 mg l^{-1}) and four sensitive (1 mg l^{-1}) *L. monocytogenes* strain	Subculture in increasing concentrations of BKC (1–10 mg l^{-1})	MIC: 1–4 mg l^{-1}	MIC increased to 5–6 mg l^{-1} for sensitive strains and to 8 mg l^{-1} for resistant ones	To et al. (2002)
GROWTH IN BKC (sub-MIC level)				
E. faecium Aus0004 and *S. aureus* HG003	Growth in sub-inhibitory concentration; 1/2 MIC	*E. faecium* Aus0004 MIC: 4 mg l^{-1}; *S. aureus* HG003 MIC: 2 mg l^{-1}	ND	Dejoies et al. (2021)
49 clonally unrelated isolates of *A. baumannii*	Growth in BKC 0.25 × MIC (10 mg l^{-1}) for 30 days - one clinical isolate only	MIC: 1–15.6 mg l^{-1} - majority at 3.9 and 7.8 mg l^{-1}	2–16-fold increase	Fernández-Cuenca et al. (2015)
205 *L. monocytogenes* food isolates	Exposure to 0.6 mg l^{-1} BKC in broth for 24 h at 37°C.	MIC range from 0.63 to 5 mg l^{-1}	MIC increased to 4 mg l^{-1}	Guérin et al. (2021)
283 *Acinetobacter* spp. strains from 97 hospitals	Repeated exposure to 1/2 MIC for 72 h	MIC: mostly 5–10 mg l^{-1}	MIC: 10–50 mg l^{-1}	Kawamura-Sato et al. (2018)
S. enterica serovar Typhimurium strains (SL1344 and 14028S)	Exposure to during use concentration - 0.015 mg l^{-1} or 0.004 mg l^{-1}	*Salmonella* SL1344 MIC: 0.03 mg l^{-1} 14028S MIC: 0.004 mg l^{-1}; MBC 0.003 and 0.008 mg l^{-1}	*Salmonella* SL1344 MIC: 3 mg l^{-1} 14028S MIC: 0.2 mg l^{-1}; MBC 8 and 20 mg l^{-1}	Knapp et al. (2015)

TABLE 1 (Continued)

Bacteria	Exposure/treatment	MIC before exposure	MIC post exposure	Reference
Burkholderia lata strain 283	Exposure to 50 mg l^{-1} BKC; 5 min or to mid-log phase at 20°C	MIC: 0.5 mg l^{-1}	No change in MIC	Knapp et al. (2013)
A. baumannii standard culture collection strain and mutants	Exposure to 32 mg l^{-1} in agar for 24 h then growth in 16 mg l^{-1}	MIC: 16 mg l^{-1}	MIC: 32 mg l^{-1}	Knauf et al. (2017)
S. aureus laboratory constructs	Pre-exposure test in 5 mg l^{-1} BKC for 60 min	MIC: 4 mg l^{-1} in parent strain; 1 mg l^{-1} in ΔnorA; 8 mg l^{-1} in norA construct; 16 mg l^{-1} in pCN38-*qacA qacR* construct	ND (increased expression of *norA* and *qacA*)	LaBreck et al. (2020)
E. coli ATCC 11775 and *E. coli* DSM 682	Sub-MIC growth 5 mg l^{-1} overnight or 20 mg l^{-1} during exponential phase	MIC: 25 mg l^{-1} for 1175 and DSM682	Exposure to 5 mg l^{-1}: 1175; MIC 25 mg l^{-1}; DSM682: MIC: 35 mg l^{-1}; exposure to 20 mg l^{-1} 1175; MIC 35 mg l^{-1}; DSM682 MIC: 45 mg l^{-1}	Langsrud et al. (2004)
E. coli CFT073	Exposure to 2 mg l^{-1} until sufficient increase in OD	MIC: 8 mg l^{-1}	ND	Ligowska-Marzeta et al. (2019)
24 coagulase-negative Staphylococci isolates (12 isolates with decreased susceptibility to CHX and 12 isolates with decreased susceptibility to BKC)	Repeated exposures (3 passages) to 1/2 MIC (range 1–4 mg l-1) BKC for 24 h at 37C	MIC range from 2 to 8 mg l^{-1} and MBC from 2 to 16 mg l^{-1}	2-fold increase in BKC MIC in 58% of CHX isolates and 83% of BKC isolates	Marzoli et al. (2021)
L monocytogenes; food and veterinary isolates	Repeated exposure to 10 mg l^{-1} BKC	MIC: 10 mg l^{-1}	MIC: 30 mg l^{-1}	Rakic-Martinez et al. (2011)
Salmonella Enteritidis NCTC13349 and one food isolate	Exposure to 1/2 MBEC (400 mg l^{-1} BKC) for 6 days every other day	MBEC: NCTC13349 800 mg l^{-1} MBEC isolate: 1600 mg l^{-1}	ND	Romeu et al. (2020)
S. maltophilia D457, and isogenic mutant D457R, overexpressing SmeDEF	Growth on agar containing 128 mg l^{-1} BKC	MIC: 128 mg l^{-1}	MIC: 256 mg l^{-1}	Sánchez et al. (2015)
Three aerobic communities from water effluent. BKCs unexposed (DP, fed a mixture of dextrin/peptone), BKCs exposed (DPB, fed a mixture of dextrin/peptone and BKCs) and BKCs enriched (B, fed only BKCs). BKC concentration 50 mg l^{-1}	Exposure of community to 50 mg l^{-1} for 4 years	ND	DPB MIC: 250 mg l^{-1}, B MIC: 460 mg l^{-1}	Tandukar et al. (2013)
P. aeruginosa ATCC27853	Repeated (10 cycles) exposure to BKC	MIC: 80 mg l^{-1}	MIC: Max 150 mg l^{-1}	Voumard et al. (2020)

Note: term *resistant/resistance* as reported in the paper but does not correspond to the definition of *resistance* used in the main document.

Abbreviations: BKC, Benzalkonium chloride, ND, not determined; TRI, triclosan.

a stable increase to BKC MIC to 150 mg l^{-1} (Voumard et al., 2020). Roedel et al. (2021) investigated ESBL/AmpC producing and no-producing *E. coli* and reported that no veterinary isolates with reduced susceptibility to BKC were found on farms, and that reduced phenotypic susceptibility to BKC and antibiotic resistance were not linked. Nordholt et al. (2021) investigated the presence of phenotypically tolerant *E. coli* subpopulations to BKC exposure and reported on persisters with reduced cell surface charge and mutations in the *lpxM* locus, which is involved in lipid A biosynthesis. In addition, they observed that although these persisters had a fitness cost in the presence of BKC, these bacteria had a better growth rate in the presence of antibiotics. Of practical significance, the study by Knapp et al. (2015) that tested the impact of bacterial short exposure (1 min) to BKC-containing products, reflecting the during use exposure of the product, demonstrated no change in antibiotic susceptibility profile.

Whilst to date, most studies are based on a pre-exposure model, a few studies stand out by exploring the impact of co-exposure of BKC with a chemotherapeutic antibiotic. Pietsch et al. (2021) investigated the impact of BKC co-exposure with some antibiotics against *P. aeruginosa* and observed a decreased activity in meropenem (although not significant), but no changes in activity when BKC was used jointly with ciprofloxacin or gentamicin. Short et al. (2021) reported on co-exposure between BKC (1–4 mg l^{-1}) and aminoglycosides against *A. baumannii* and observed antagonism between BKC and aminoglycosides whereby gentamicin bactericidal activity was severely reduced. In addition, this study showed that BKC exposure increased the frequency of emerging *A. baumannii* mutants with reduced susceptibility to aminoglycosides following decreased intracellular accumulation of the antibiotics. Although of significance in this in vitro investigation, the authors stated that the likelihood of such co-exposure in practice was likely to be rare (Short et al., 2021). However, an earlier study by Morita et al. (2003) did not show any difference in norfloxacin efficacy in *P. aeruginosa* when BKC was added at 20 mg l^{-1} (0.33 × MIC) to the growth media.

Bacterial stress response following BKC exposure

The expression of stress response as a result of BKC exposure has been mentioned in a few studies. Ceragioli et al. (2010) looked at gene expression in *Bacillus cereus* exposed to BKC (0.5 to 7.0 mg l^{-1}) during mid-exponential growth. A common response to BKC exposure included expression of genes involved in the general and oxidative stress responses. A specific response to BKC exposure included expression of genes involved in fatty acid metabolism. In *Listeria monocytogenes*, exposure to BKC 50 mg l^{-1} for 10 min resulted in elevated c-di-GMP levels and the synthesis of an exopolysaccharide that promoted cell aggregation, inhibited motility in semi-solid media and desiccation (Chen et al., 2014). Food isolates of *L. monocytogenes* exposed to 10 mg l^{-1} BKC (although exposure was not explicit in the paper) resulted in enrichment of bacterial genomes with genes involved in alkaline and oxidative stress (Pasquali et al., 2018). Peyrat et al. (2008) demonstrated that deletion of *sigB*, a gene involved in bacterial stress response, affected the ability of *L. monocytogenes* to survive lethal concentration (40 mg l^{-1}) of BKC, although the deletion of *sigB* did not affect BKC MIC. Similarly, van der Veen and Abee (2010) showed *sigB* deletion decreased the viability planktonic and sessile *L. monocytogenes* exposed to BKC (20 mg l^{-1} for 15 min). Conversely, overexpression of *sigB* in *L. monocytogenes* was associated with a decreased susceptibility to BKC (10 mg l^{-1} for 5 min) (Tamburro et al., 2015). Investigating bacterial cell injuries caused by food processing method, Siderakou et al. (2021) showed that 6 h incubation in BKC at 100 mg l^{-1} at 4°C or 20°C did not result in the formation of injured *L. monocytogenes* subpopulations, but significantly affected their survival. The exposure of *A. baumannii* to 6 mg l^{-1} BKC resulted in the expression of 227 genes involved in bacterial fitness and 335 genes involved with cell envelope maintenance, drug efflux, proteostasis and oxidative stress defence (Knauf et al., 2017). In *Klebsiella pneumoniae*, the Cpx envelope stress response system was shown to play a role in response to BKC exposure (Srinivasan et al., 2012). Exposure of growing (mid-log growth phase) *Desulfovibrio vulgaris* Hildenborough to BKC (1.25 mg l^{-1}) significantly changed gene expression (103 upregulated and 95 downregulated), mostly genes involved with cell envelope biogenesis, outer membrane, metabolism, energy production and conversion, translation, ribosomal structure and biogenesis, although the clinical significance of such finding was unclear (Lee et al., 2010). Merchel Piovesan Pereira et al. (2020) reported the overexpression of several chaperones and cochaperonins, such as *dnaK*, *dnaJ*, *groL*, *groS*, *htpG*, *hscA*, *cpxP* and *clpB* in *E. coli* following exposure to BKC 3.63 mg l^{-1} for 30 min.

Role of efflux in decreased susceptibility to BKC

The presence of efflux pump genes or expression of efflux has been implicated as a mechanism responsible for a decrease in MIC, regardless of bacterial genera, in 80% of the retained studies (Figure 3). Of those, 42% (50/120) mentioned Qac-based efflux pumps. Carriage of efflux pump genes was observed to be widespread in

Gram-positive bacteria including staphylococci (Kaatz and Seo, 2004 ; Kaatz et al., 2005; Bjorland et al., 2005; Correa et al., 2008; Couto et al., 2008, 2013, 2015; DeMarco et al., 2007; Falcão-Silva et al., 2009; Costa et al., 2010, 2013; Furi et al., 2013; Heir et al., 1999a, 1999b; Ho & Branley, 2012; Wong et al. 2013; Ignak et al., 2017; Lee et al., 2020; Marzoli et al., 2021; Morrissey et al., 2004; Shamsudin et al., 2012; Sidhu et al., 2002; Slifierz et al., 2015; Worthing et al., 2018; Zhang et al., 2012; Zmantar et al., 2011; Kroning et al., 2020), *Lactococcus* spp. (Fernández Márquez et al., 2014), *Enterococcus* spp. (Fernández Márquez et al., 2014; Alotaibi et al. 2017; Ignak et al., 2017; Morrissey et al., 2004), *Lactobacillus* spp. (Fernández Márquez et al., 2014) and *Bacillus* spp. (Fernández Márquez et al., 2014), and Gram-negative bacteria such as *K. pneumoniae* (Morrissey et al., 2004; Abuzaid etal., 2012; Wand et al., 2015; Wang et al., 2008), *E. coli* (Paulsen et al., 1993; Hansen et al., 2007; Kumar and Doerrler, 2014; Deus et al., 2017; Fernández Márquez et al., 2014; Guo et al., 2015; Bay et al., 2017; Jiang et al., 2017; Puangseree et al., 2021; Wang et al., 2008), *P. aeruginosa* (Stoitsova et al., 2008; Amsalu et al., 2020; Gholamrezazadeh et al., 2018; Goodarzi et al., 2021; Namaki et al., 2022; Romão et al., 2011), *Enterobacter* spp. (Boutarfi et al., 2019; Fernández Márquez et al., 2014; Morrissey et al., 2004; Wang et al., 2008), *Helicobacter* spp. (Fernández Márquez et al., 2014), *P. mirabilis* (Jiang et al., 2017), *L. monocytogenes* (Soumet et al., 2005; Romanova et al., 2006; Müller et al., 2013, 2014; Bland et al., 2021; Cherifi et al., 2020; Chmielowska et al., 2021; Cooper et al., 2021; Dutta et al., 2013; Gray et al., 2021; Kremer et al., 2017; Meier et al., 2017; Mereghetti et al., 2000; Møretrø et al., 2017; Roedel et al., 2019; Tamburro et al., 2015), *Citrobacter freundii* (Kücken et al., 2000), *Stenotrophomonas maltophilia* (Kücken et al., 2000; Wang et al., 2008), *A. baumannii* (Srinivasan et al., 2011; Khosravi et al., 2021; Liu et al., 2017; Rajamohan et al., 2010a, 2010b; Wang et al., 2008), *Salmonella* spp. (Heir et al., 1998; Morrissey et al., 2004; Aksoy et al., 2019), *Yesinia enterocolitica* (Bengoechea and Skurnik, 2000), *Legionella pneumophila* (Ferhat et al., 2009) and *Flavobacterium* spp. (Wang et al., 2008). However, the role of BKC in the dissemination of these genes was not reported, or when mentioned, speculative. Morrissey et al. (2004) investigated the association of an elevated BKC MIC with the presence of *qac* genes *qacI*, *qacE* and *qacK* in 3319 clinical isolates of various bacterial genera; no association was found, although the definition of an elevated MIC (ECOFF's value) was arbitrary. Other studies have looked at bacterial construct to study the impact of a particular efflux pump, resulting at time in a decreased bacterial susceptibility to BKC (Braga et al., 2011; Chittrakanwong et al., 2021; Guérin et al., 2016; Huang et al., 2004; Kaatz & Seo, 1995; LaBreck et al., 2020; Li et al., 2008; Maseda et al., 2009; Matsuo et al., 2014; Nishino et al., 2006; Nordholt et al., 2021; Paulsen et al., 1995; Pereira et al., 2021; Rajamohan et al., 2010a; Srinivasan et al., 2009, 2014).

Decrease in antibiotic susceptibility has been associated with efflux in Gram-positive bacteria, for example, staphylococci (Bjorland et al., 2005; Correa et al., 2008; Marzoli et al., 2021; Zhang et al., 2011; Zmantar et al., 2011), *Lactococcus* spp. (Fernández Márquez et al., 2014) and *Lactobacillus* spp. (Fernández Márquez et al., 2014), and Gram-negative bacteria such as *E. coli* (Fernández Márquez et al., 2014; Pereira et al., 2021; Puangseree et al., 2021), *Enterobacter* spp. (Boutarfi et al., 2019), *P. aeruginosa* (Amsalu et al., 2020; Namaki et al., 2022; Romão et al., 2011), *Helicobacter* spp. (Fernández Márquez et al., 2014), *Campylobacter* spp. (Mavri & Smole Možina, 2013), *A. baumannii* (Khosravi et al., 2021; Srinivasan et al., 2009) and *L. monocytogenes* (Gray et al., 2021; Kremer et al., 2017). In six studies, both a change in efflux and membrane properties were associated with decreased susceptibility to BKC (Bischofberger et al., 2020; Fernández-Cuenca et al., 2015; Langsrud et al., 2004; Mavri & Smole Možina, 2013; Nagai et al., 2003; Pereira et al., 2021). A few investigations looked *at ad litteram* 'inducible resistance' (as defined in this review inducible reduced susceptibility) when bacteria were exposed to BKC. Beier et al. (2014) showed that BKC was associated with low induced resistance (compared to other biocides) in *P. aeruginosa* veterinary isolates at rates of 10–13%; here, inducible resistance was defined as growth of the organism above the MIC. Morita et al. (2003) showed indirectly the induction of efflux in *P. aeruginosa* in the presence of BKC (6 $mg\,l^{-1}$); *P. aeruginosa* was able to grow in the presence of both BKC (6 $mg\,l^{-1}$) and norfloxacin (1 $mg\,l^{-1}$), whilst growth in the presence of norfloxacin (1 $mg\,l^{-1}$) alone was inhibited.

With *S. aureus* harbouring a luciferase QacR (efflux pump gene repressor) construct, Galluzzi et al. (2003) showed that BKC (1 $mg\,l^{-1}$) exposure repressed the expression of QaCR. A follow-up study showed that BKC (12 $ng\,ml^{-1}$) induces the light emission of the luciferase QacR construct by two-fold, demonstrating the low threshold impact of BKC-induced response (Galluzzi et al., 2004). Likewise, Grkovic et al. (2003) showed that BKC (1 $mg\,l^{-1}$) was a moderate inducer of QacA.

Braga et al. (2011) observed that efflux gene expression (*qacZ*) in *Enterococcus faecalis* was not inducible by BKC, although *qacZ* expression was associated with a decreased susceptibility in *E. faecalis*. Merchel Piovesan Merchel Piovesan Pereira et al. (2020) showed

that long exposure (8–12 h) to BKC 3.63 mg l^{-1} led to the expression of a biofilm phenotype in *E. coli*. In *Stenotrophomonas maltophilia*, overexpression of smeDEF (efflux pump) was associated with a 2-fold decrease in BKC MIC (from 128 to 256 mg l^{-1}) and a reduced susceptibility to quinolones and chloramphenicol (Sánchez et al., 2015). Bioinformatics predicted BKC should induce *smeDEF* expression leading to transient *S. maltophilia* resistance to antibiotics since BKC binds to SmeT, the repressor of *smeDEF*. However, phenotypic assays showed that this was not the case (Sánchez et al., 2015). Chittrakanwong et al. (2021) observed that the addition of BKC (2–8 mg l^{-1}) during the exponential growth phase of *S. maltophilia* resulted in derepressing MfsR activity, a QAC-sensing regulator controlling the expression of the major facilitator superfamily efflux transporter mfsQ, enabling bacterial growth in 75 mg l^{-1} BKC.

Other mechanisms involved in decreased BKC susceptibility

Other studies have associated a decrease in BKC susceptibility with a change in bacterial cytoplasmic membrane composition (Bisbiroulas et al., 2011; Bischofberger et al., 2020; Ceragioli et al., 2010; Guérin-Méchin et al., 2004; Jennings et al., 2017; Maertens et al., 2020; Noll et al., 2020; Voumard et al., 2020), outer membrane protein (Ishikawa et al., 2002; Pereira et al., 2021; Srinivasan et al., 2012; Tabata et al., 2003) or outer membrane (Chen et al., 2014; Ishikawa et al., 2002; Joynson et al., 2002; Loughlin et al., 2002; Manniello et al., 1978; Nordholt et al., 2021). One study attributed a decrease in BKC susceptibility associated with an increase in MIC to some antibiotics to a combination of mutations and efflux (Kim et al., 2018). Another study on *Acinetobacter baumannii* suggested that decreased susceptibility to BKC is associated with ribosomal protein mutations that protect *A. baumannii* against BKC-induced protein aggregation (Knauf et al., 2017).

BKC exposure and impact on biofilm

Only a few studies investigated the effect of BKC exposure at sub-MIC concentration on biofilm formation and evidence from different studies is at time contradictory. BKC was shown to induce biofilm formation in *Staphylococcus epidermidis* (Houari & Di Martino, 2007), *K. pneumoniae* (Elekhnawy et al., 2021) and *L. monocytogenes* (Bonneville et al., 2020; Piercey et al., 2017), or to increase biofilm biomass in *E. coli* (Machado et al., 2012; Yu et al., 2021), *P. aeruginosa* (Machado et al., 2012) and *A. baumannii* (Rajamohan et al., 2009). Others have however shown that BKC exposure decreases bacterial ability to form biofilms in *S. aureus* (Buzón-Durán et al., 2017) and in *L. monocytogenes* (Ortiz et al., 2014a). BKC pre-exposure also decreased susceptibility of *Salmonella enterica* (Mangalappalli-Illathu & Korber, 2006) and *L. monocytogenes* (Ortiz et al., 2014a) biofilms to BKC. Different concentrations of BKC may impact differently on biofilm formation. Chaieb et al. (2011) observed that BKC (1 mg l^{-1}) treatment induced biofilm formation in *Staphylococcus epidermidis*, whereas higher concentrations (2, 3, 4 and 5 mg l^{-1}) decreased biofilm formation altogether. Pang et al. (2020) reported no change in *Salmonella* Enteritidis biofilm susceptibility to BKC 100 or 200 mg l^{-1} following biofilm pre-exposure to 20 mg l^{-1} BKC for 5 days.

A few studies have looked at the impact of BKC treatment on complex biofilm communities. Gray et al. (2021) showed that bacterial isolates from water treatment plants that were able to grow on 250–500 mg l^{-1} BKC accounted for 0.2% of the overall culturable communities and were mainly *Pseudomonas* spp. A high proportion of these isolates with colistin resistance were observed, but no direct link between BKC insusceptibility and colistin resistance was demonstrated. Tandukar et al. (2013) observed a decrease in diversity of a water effluent microcosm following exposure to BKC 50 mg l^{-1} for 4 years. In addition, an increased BKC MIC (<2-fold) of surviving bacteria was observed. Bastian et al. (2009) analysed a bacterial microcosm that was exposed to a product containing10–25% BKC and concluded a change in microflora occurred as a result of selection over a 3-year application of the antifungal product. Forbes et al. (2017) exposed domestic drain biofilms daily with BKC-containing products (100 mg l^{-1} BKC) for 6 months and observed an enrichment of bacteria, particularly pseudomonads, able to grow at BKC concentrations up to 100 mg l^{-1}. Although a change in antibiotic susceptibility profile was observed, no bacteria became clinically resistant to the antibiotics tested. In addition, bacteria with reduced BKC and antibiotic susceptibility were susceptible to higher BKC concentrations, and BKC formulation significantly enhanced this effect. Chacón et al. (2021) observed a decrease in alpha diversity values and an expansion of the relative abundance of Alphaproteobacteria in a complex microcosm from a domestic water treatment plant following in vitro exposure to 10 mg l^{-1} BKC for 96 h. In addition to the change in bacterial community diversity, a significant increase in the *qacE*/*qacEΔ1* genes was observed.

BKC exposure and gene maintenance transfer

Only a very few studies reflected on gene transfer conferring a reduced susceptibility to BKC. None of them investigated the impact of BKC on gene transfer or maintenance. Bjorland et al. (2001) described the occurrence of a small QAC resistance plasmid in 3 out of 31 veterinary *S. aureus* isolates belonging to the same clone suggesting a lateral transfer of plasmid-borne QAC resistance. The authors speculated that maintenance of the plasmid results from the continuous use of BKC-based preparations, but they did not offer tangible data supporting their assertion. In addition, isolates carrying the plasmid showed only a 2–3-fold increased BKC MIC (2.5–3 $mg\,l^{-1}$).

Harrison et al. (2020) observed an increase in the abundance of *sul1* and Bla_{TEM} in a complex microcosm exposed to BKC (0.0001–0.5 $mg\,l^{-1}$) for 14 days. BKC was reported to positively select bacterial resistance to ciprofloxacin and sulphamethoxazole, but negatively select for resistance to ampicillin, streptomycin, rifampicin, erythromycin, trimethoprim and tetracycline (to note the antibiotic clinical resistance determination was not standard). The authors concluded that widespread use of BKC might impact bacterial resistance profiles in the environment but called for a better understanding of the mechanisms of resistance and the active expression of resistance genes in the environment. Although this in vitro study enabled to investigate the use of a specific antimicrobial against a complex microcosm, in the environment, the impact of a specific compound on a microcosm is difficult to ascertain because of the documented number of compounds, including antibiotics (Felis et al., 2020; Rodriguez-Mozaz et al., 2020) particularly found in water effluents.

Yamamoto et al. (1988) showed the occurrence of *S. aureus* transconjugants with an elevated BKC MIC (6.25 $mg\,l^{-1}$) compared to the recipient strain (MIC of 3.13 $mg\,l^{-1}$) but not to the donor (MIC of 12.5 $mg\,l^{-1}$). However, the MIC determination did not use a standard protocol and gene(s) transferred were not identified. Correa et al. (2008) proposed that *qac* genes are horizontally spread amongst different clones based on cluster analysis of 21 isolates of *Staphylococcus haemolyticus*. BKC MIC ranged between 1 and 8 $mg\,l^{-1}$, but a number of isolates were resistant to gentamicin (21), erythromycin (15), ciprofloxacin (18), chloramphenicol (7) and tetracycline (1). The impact of BKC exposure to the *qac* genes dissemination was not studied.

Jiang et al. (2017) reported that QAC resistance genes were present amongst 52 *Proteus mirabilis* isolates from cooked meats (BKC MIC ranged from 4 to >32 $mg\,l^{-1}$, but the majority of isolates had an MIC of 24 $mg\,l^{-1}$). *qacH* was associated with non-classic class 1 integrons located on conjugative plasmids. The impact of BKC exposure to the *qacH* dissemination was not studied. In another study with 179 *E. coli* food isolates (BKC MIC ranged from 4 to 64 $mg\,l^{-1}$, with 44% isolates showing an MIC of 32 $mg\,l^{-1}$), Jiang et al. (2017) showed that *qacH*-associated integrons located on 100 kb plasmids in two isolates could be transferred to an *E. coli* recipient. The impact of BKC exposure to the *qacH* dissemination was not studied. Katharios-Lanwermeyer et al. (2012) showed that heavy metal (cadmium) and BKC could be co-selected during conjugation with *Listeria* spp. harbouring the BKC resistance cassette *bcrABC*. The paper mentioned that BKC-'resistant' strains have a higher ability for conjugation, but the MIC protocol is not described, and 'resistant' strains are not defined. Kücken et al. (2000) showed that BKC MIC in a naive *E. coli* (MIC of 20 $mg\,l^{-1}$) increased to 80 $mg\,l^{-1}$ following transformation with a plasmid containing an integron *qacE*. However, there was no correlation between increased BKC MIC and the presence of *qacE* or *qacEΔ1*.

BKC and in situ investigations

Only one study to date has looked at the impact on household microflora of BKC-containing products; a liquid kitchen spray containing 0.08% alkyl dimethyl benzyl ammonium chlorides and 0.02% alkyl benzyl ammonium chlorides, and an 'all-purpose' surface cleaner containing 2.7% alkyl benzyl ammonium chlorides; together with an antimicrobial handwashing soap containing 0.2% triclosan (Carson et al., 2008). In this in situ study, the authors used an ad hoc definition of resistance. After one year of assigned product usage, bacterial isolates with high BKC MICs were more likely to have high MICs for triclosan and be resistant to one or more antibiotics. The change in antibiotic susceptibility profile was random and there was no follow-up to investigate the stability of these profiles or the clinical significance of these findings. In addition, it is unclear whether the change in antibiotic susceptibility profile was driven by the BKC products only.

BKC products or formulations

Overall, only 15% (31/212) of the retained studies tested a product formulation containing BKC. None of the studies referred to a Chemical Abstracts Service (CAS) number. Not all studies that used formulations provided details of the content of the formulation. Specific product information might be difficult to find since some products are

no longer available. Nevertheless, when a BKC formulation or product was used, it was clear that the formulation achieved a better efficacy than BKC used on its own. Cowley et al. (2015) repeatedly exposed isolates (different genera) to formulations with and without BKC. MIC and MBC were 11-fold lower for formulations in general. Exposure to BKC formulations did not increase MIC or MBC to the same extent as formulations without BKC. In addition, adapted strains were not stable. Lee et al. (2020) observed an increased in BKC MIC in *S. epidermidis* (MIC: 0.00098 mg l^{-1} for methicillin-susceptible *S. epidermidis* and 0.00231 mg l^{-1} for methicillin-resistant *S. epidermidis*) isolated from patient treated with Xalatan (0.005%; using BKC as a preservative; the duration of treatment was not disclosed). Isolates with high BKC MIC harboured efflux encoding genes *qacC/smr*. Kawamura-Sato et al. (2008) showed that *Acinetobacter* spp. isolates adapted to BKC and showing increased MIC were not able to grow in the presence of an in-use concentration of BKC (2000 mg l^{-1}). Likewise, Condell et al. (2012) showed that adapted *S. enterica* isolates with increased BKC MIC could not grow in a 50% dilution of a BKC-containing product. Bland et al. (2021) showed that 45/48 *L. monocytogenes* environmental isolates collected from produce handling and processing facilities in the USA had their growth characteristic severally affected by the presence of 1.56 mg l^{-1} of BKC product. Two of three isolates that were not affected by BKC harboured the *bcrAB* cassette suggesting efflux was responsible for the unaltered growth of these isolates in BKC. Forbes et al. (2016) reported that exposure to BKC formulation was more likely to increase antibiotic susceptibility than adaptation to the simple BKC aqueous solution. Worthing et al. (2018) reported elevated BKC-product MIC and MBC in staphylococcal veterinary isolates, but all MBC values were well below the BKC-product recommended usage concentration. Avrain et al. (2003) reported that antibiotic-resistant *Campylobacter* spp. veterinary isolates did not have a reduced susceptibility to the BKC-product tested. Following routine use of a BKC-based product, Peyrat et al. (2008) isolated *Campylobacter* strains with decreased BKC-MIC but with no change in antibiotic susceptibility profile. Nhung et al. (2015) reported, however, a weak correlation between increased BKC-product MIC and decreased antibiotic susceptibility in *E. coli* and *S. enterica* following stepwise exposure to a BKC product, although none of the changes were clinically significant. Stickler and Thomas (1976) reported a correlation between increased BKC-product MIC and CHX product in *Providencia stuartii*. Knapp et al. (2015) tested the efficacy and impact of exposure to the 'during use' concentration of BKC product (shampoo) against industrial isolates of *P. aeruginosa*, *B. cepacia* and *K. pneumoniae*, and two *S. enterica* serovar Typhimurium, and reported no changes in susceptibility profile to BKC or antibiotics. Beier, Andrews et al. (2021), Beier, Byrd et al. (2021) investigated the susceptibility of *S. aureus* (Beier, Andrews, et al., 2021; Beier, Byrd et al., 2021) and *C. jejuni* (Beier, Andrews, et al., 2021; Beier, Byrd et al., 2021) isolates to BKC and other biocides as well as antibiotic susceptibility profile. Whilst some isolates showed elevated MIC to several biocides and resistance to some antibiotics, they remained susceptible to a BKC product (Beier, Andrews, et al., 2021; Beier, Byrd et al., 2021). Bassani et al. (2021) observed no differences in bacterial susceptibility to BKC or antibiotics in *S. Heidelberg* isolates over a 10-year period.

When a complex microcosm was investigated, Forbes et al. (2017) exposed domestic (kitchen) drain biofilms daily with BKC-containing products (100 mg l^{-1} BKC) for 6 months and observed an enrichment of bacteria, particularly pseudomonads, able to grow at concentrations up to 100 mg l^{-1} BKC. Although a change in antibiotic susceptibility profile was observed, no bacteria became clinically resistant to the antibiotics tested. In addition, bacteria with reduced BKC and antibiotic susceptibility were susceptible to higher BKC concentrations. Likewise, Bastian et al. (2009) reported a decrease in bacterial diversity in a microcosm that was exposed to Devor Mousse® that contains10–25% BKC and concluded the change in microflora resulted from selection following the 3-year application of the antifungal product. Tandukar et al. (2013) observed a decrease in the diversity of a water effluent microcosm following exposure to BKC 50 mg l^{-1} for 4 years. In addition, an increase in BKC MIC (<2-fold) of surviving bacteria was observed.

BENZETHONIUM CHLORIDE AND BACTERIAL 'RESISTANCE'

Benzethonium chloride (CAS Number 121-54-0) is a synthetic quaternary ammonium compound with a broad spectrum of activity used for preservation, antisepsis and disinfection. BZT has been less studied than BKC and there are only six relevant studies on bacterial 'resistance' to BZT (Table S2). Most of these publications (four of six) deal with food bacteria, notably *Listeria monocytogenes* (Casey et al., 2014; Collins et al., 2012), *Salmonella enterica* serovar Typhimurium (Chen et al., 2007) and *E. coli* (Deus et al., 2017). One study investigated *Streptococcus mutants* (Oyanagi et al., 2012) and another industrial isolates of *Burkholderia* spp (Rushton et al., 2013).

In these studies, measurement of activity was based mainly on the use of a microdilution broth (Collins et al., 2012; Deus et al., 2017; Rushton et al., 2013) but not necessarily following the standard CLSI protocol.

Only three publications investigated the effect of pre-exposure to BZT. Collins et al. (2012) grew *L. monocytogenes*

in different concentrations of BZT and investigated BZT effects on different *L. monocytogenes* mutants. Their study was not focussed on BZT but used BZT as one of the biocides investigated. They concluded that mutants with a deletion in the *liaS* gene that encodes for intramembrane-sensing histidine kinases, which are involved in stress response, were more tolerant to BZT than the wild-type bacteria. This publication did not look at cross-resistance to chemotherapeutic antibiotics.

Rushton et al. (2013) exposed *Burkholderia cenocepacia* complex (Bcc) subcultures to increasing concentrations of BZT in an agar-based protocol. BZT at 1 $g l^{-1}$ failed to inhibit the growth of 93% (77/83) of Bcc strains. They observed that 14 Bcc strains (predominantly *B. cenocepacia*) showed a high tolerance for BZT, with MBCs up to 10 times >1 $g l^{-1}$. There was no cross-resistance to other preservatives such as isothiazolinones or chemotherapeutic antibiotics. The adapted Bcc strains displayed altered growth pattern with longer lag phase and shorter culture doubling time.

Casey et al. (2004) studied gene regulation (transcriptome and RNA-seq analysis) following incubation of *L. monocytogenes* 6179 with BZT 4 $mg l^{-1}$ at 14°C to early stationary phase. They identified approximately 600 genes that showed a 4-fold or greater change in relative expression in the BZT-treated sample compared to the control. The functions of genes that were upregulated concern chemotaxis, fatty acid metabolism, flagellar assembly, cobalamin (Vitamin B12) biosynthesis, peptidoglycan biosynthesis and phosphotransferase system. Of interest is the upregulation of a multidrug transporter pump: 4-fold increase in *qacH* and 3-fold increase in *ykkC* and *lmrB*, which encode efflux transporters. They did not study changes in MIC/MBC and antibiotic susceptibility profile.

Oyanagi et al. (2012) performed a suspension test with 0.1 $g l^{-1}$ BZT against *S. mutans* MT8148 and *Streptococcus sobrinus* 6715 and observed only a 50% reduction in bacterial number within a 10-s contact time. They did not study antibiotic susceptibility profile.

Deus et al. (2017) investigated the susceptibility of 174 human and veterinary isolates of extended-spectrum beta-lactamases *E. coli* to several biocides including BZT. The broth microdilution performed to determine susceptibility profile was not standard, but custom made. The MIC distribution was unimodal and presented a narrow range. The isolates showing elevated MIC to BZT ($n = 6$; 32 µg/ml) harboured a number of efflux pump genes involved in QAC tolerance; these included *emrE*, *mdfA*, *sugE*, *sugE*, *oqxA*, *oqxB*, *qacE*, *qacEΔ1*, *qac*F, *qacG* and *qacH*.

Out of the six peer-reviewed papers on BZT, only one study investigated the effect of BZT exposure on antibiotic susceptibility changes. The study by Rushton et al. (2013) managed to produce stable mutants with increased BZT MBC through the use of stepwise training. No change in antibiotic susceptibility was observed. Casey et al. (2014) investigated *L. monocytogenes* response to BZT (4 $mg l^{-1}$) exposure and notably the upregulation of efflux pump genes. The significance of gene upregulation is unclear since no change in susceptibility data was provided. Correlation between BZT usage, and efflux gene carriage and dissemination, has not been made in the only study available (Deus et al., 2017). No information on the impact of BZT on transfer or maintenance of resistant genes could be found.

CHLOROXYLENOL AND BACTERIAL 'RESISTANCE'

Chloroxylenol (CAS number 88–04-0 check) also known as para-chloro-meta-xylenol (PCMX) is a halophenol used for disinfection and antisepsis. It is a membrane-active agent with a broad spectrum of activity. Studies published in the peer-reviewed literature on chloroxylenol (CHO) and antimicrobial 'resistance' (Table S3) concern a wide range of environments including healthcare and veterinary (Gilbert et al., 2002; Johnson et al., 2002; Tambe et al., 2011; Young et al., 2011), home (Cole et al., 2003; Moken et al., 1997), food (Collins et al., 2012) and industry (Lear et al., 2002, 2006) (Table S3). Several studies investigated the impact of bacterial biofilms on biocide efficacy (Das et al., 1998; Gilbert et al., 2002; Johnson et al., 2002). These studies did not necessarily focus on CHO but use CHO as an additional biocide amongst other actives.

Several studies measure biocide susceptibility as MIC or/and MBC using a microdilution broth based on standard protocols (Collins et al., 2012; Gilbert et al., 2002; Johnson et al., 2002; Tambe et al., 2011). Others used ad hoc protocols which makes the comparison of susceptibility data difficult (Cole et al., 2003; Conroy et al., 2010; De Majumdar et al., 2015; Young et al., 2011).

The study by Cole et al. (2003) is an in situ randomized trial focussing on the home environment. A total of 1268 isolates were collected following the use of household products in selected homes and tested for their susceptibility to several biocides and antibiotics. MIC for CHO ranged from 62 to 250 $mg l^{-1}$. Antibiotic testing was conducted using a standard CLSI test. Overall, there was no meaningful correlation between antibiotic resistance in Gram-positive and Gram-negative human pathogens. Collins et al. (2012) grew *L. monocytogenes* in different concentrations of CHO and investigated CHO effects on different *L. monocytogenes* mutants. They concluded that mutants with a deletion in the *liaS* gene, which encodes for intramembrane-sensing histidine kinases involved in stress response, were more tolerant to CHO than the wild

type. This publication did not look at cross-resistance. De Majumdar et al. (2015) observed that overexpressing *ramA* in *K. pneumoniae* resulted in increased tolerance to CHO and other compounds, including chemotherapeutic antibiotics, probably resulting from a change in membrane lipopolysaccharide composition. Conroy et al. (2010) investigated potential substrates of two RND-type transport systems. The authors use the Biolog system to measure susceptibility and concluded these pumps confer CHO 'moderate' resistance; this level of resistance was not defined. Overall, this study showed that CHO was a substrate for these pumps that may contribute to an elevated MIC to CHO. Likewise, Moken et al. (1997) related the presence of *acrAB* to decrease susceptibility to CHO in *E. coli*.

Das et al. (1998) and Gilbert et al. (2002) investigated the impact of biofilms on decreased CHO susceptibility in *Staphylococcus epidermidis* NCTC 11047 and a mucoid isolate of *E. coli*. Measurement of biocide susceptibility was based on optical density (OD) measurement. There is no standard test for that type of biofilm testing. Das et al. (1998) observed that CHO was equally efficacious against planktonic and sessile bacteria, whilst Gilbert et al. (2002) concluded that pre-exposure of biofilms to sub-lethal concentrations of CHO did not impact biofilm changes in susceptibility to the biocide. A study from Young et al. (2011) merely looked at susceptibility of veterinary isolates to CHO using ad hoc methodology and did not measure susceptibility to chemotherapeutic antibiotics.

Perhaps, the most interesting articles are those of Lear et al. (2002, 2006) and Tambe et al. (2011). Lear et al. (2002) investigated CHO resistance in industrial isolates where CHO is manufactured. In addition, a range of protocols were used to explore the development of bacterial resistance to CHO, including the heavy inoculum approach, exposing 10^9 CFU spread on a tryptone soya agar plate containing 100–500 mg l^{-1} CHO, and repeated exposure to sub-MIC (50 and 100 mg l^{-1}) of CHO. They observed that industrial isolates tolerant (MIC > 500 mg l^{-1}) to CHO were mostly *Pseudomonas aeruginosa* (24 isolates), *Alcaligenes xylosoxidans* (1 isolate) and *Pseudomonas fluorescens* (1 isolate). Five isolates of *Pseudomonas stutzeri* were identified with a CHO MIC range of 300–500 mg l^{-1}, whilst a culture collection strain had an MIC of 200–220 mg l^{-1}. The observed CHO tolerance was stable without the presence of CHO. The heavy inoculum or the repeated exposure approaches did not yield any resistant *Ps. stutzeri* to CHO. In addition, using a standard suspension test, Lear et al. (2002) showed that the CHO tolerant *Ps. stutzeri* was as susceptible to CHO at 250 mg l^{-1} as the culture collection strain. Follow-up investigation on bacterial tolerance to CHO could not link high bacterial tolerance (measured based on MIC) to bactericidal efficacy (at 10 mg l^{-1} of CHO) or significant change in antibiotic susceptibility profile (Lear et al., 2006).

Tambe et al. (2011) investigated the effect of CHO sub-MIC exposure on increased insusceptibility in *S. epidermidis*. They observed no changes in CHO MIC or MBC (remaining at 250 mg l^{-1}) after 20 passages.

Only two studies investigated the effect of CHO exposure on bacterial cross-resistance to antibiotics (Cole et al., 2003; Lear et al., 2006). No significant changes were observed and relevance to clinical significance was not explored. The three in vitro studies (Johnson et al., 2002; Lear et al., 2002; Tambe et al., 2011) that investigated pre-exposure to a sub-lethal concentration of CHO did not find evidence of a change in MIC or MBC. The study investigating CHO resistance in industrial isolates which were likely to be continuously exposed to CHO did not find any evidence of decrease in bacterial susceptibility to CHO (Lear et al., 2002).

CONCLUSION

The use of products containing BKC, BZT and CHO has been viewed as a concern for potential development of decreased bacterial susceptibility to these biocides and/or chemotherapeutic antibiotics. A total of 3655 scientific papers mentioning these biocides were analysed. The vast majority did not make the inclusion criteria and overall, very few relevant papers specifically dealing with antimicrobial resistance were retained. Most of the studies related to BKC, with 212 papers retained, and very few were relevant to CHO (12) and BZT (6).

One evident issue with the scientific literature was the lack of standardization of the method used to measure susceptibility to biocides and antibiotics. For example, with the literature on BKC the majority of studies 161/212 (76%) reports MIC data which does not reflect product usage in practice. Only 27% of these (43/161) used a standardized protocol, principally the CLSI broth microdilution method or agar dilution-based method. In addition, only 10% (21/212) of the retained studies provided information on MBC and only 10% (22/212) performed bactericidal tests such as a suspension test. The use of an increase in MIC as a sole indicator of resistance has been criticized as not reflecting product usage in practice, but to just provide an indication that a biocide can alter a bacterial phenotype (Maillard et al., 2013; Russell & McDonnell, 2000). It has long been argued that a change in MIC does not necessarily indicate a bacterium will be resistant to a biocide, particularly when one considers that the concentration used in a product is often considerably higher (>1000 fold) than the MIC (Maillard et al., 2013; Russell & McDonnell, 2000). To

add some perspective, the handbook of Pharmaceutical Excipients (Rowe et al., 2009) reports BKC in-use concentrations for the preservation of pharmaceutical preparations of 100–200 mg l^{-1} and as low as 20 mg l^{-1} for otic formulations. In practice, BKC-based soap products contain between 1 and 10 g l^{-1} BKC, BKC-based sanitizing products contain BKC concentrations ranging from 200 to 400 mg l^{-1}, whilst hospital disinfectants typically contain 1.2–2.4 g l^{-1} BKC. A BKC concentration of 1 g l^{-1} represents the low ends of the active eligibility monograph ranges for use in antiseptic products covered by the OTC antiseptic. Increases in MIC following repeated exposure to the same BKC concentration (Table 1) rather than stepwise training (repeated exposure in increasing BKC concentration), resulted in BKC MIC < 50 mg l^{-1} in single bacterial species (Kawamura-Sato et al., 2008). Two recent manuscripts investigated the effect of co-exposure of BKC with an antibiotic and demonstrated a reduced antibiotic activity (Pietsch et al., 2021; Short et al., 2021). Whilst the science is interesting, the likelihood of co-exposure occurring in practice is remote.

Clinical, veterinary and environmental isolates showing an elevated BKC MIC have been shown to carry many efflux pump genes. Correlation between BKC usage, and efflux gene carriage and dissemination, has not been made in any in vitro studies. The clinical significance of a decreased antibiotic susceptibility in isolate with a decreased susceptibility to BKC has not been well studied. Where clinical resistance to antibiotics was observed, a direct correlation with BKC usage was not established. Furthermore, there is no information on the impact of BKC on transfer or maintenance of resistance genes. Bacterial isolates with a decreased BKC susceptibility have not been shown to be more virulent (in animal assays) and increased fitness (measured by growth rate) of isolates with a decreased BKC susceptibility, when studied, was shown, perhaps not surprisingly, to be elevated in the presence of BKC. Amongst the 230 retained papers, only one in situ paper raised a concern that repeated BKC exposure selects for bacteria leading to a clinical change in antibiotic susceptibility. It is however unclear whether the change in antibiotic susceptibility profile was driven by the BKC products only as the handwashing product used in this study contained triclosan, which is known to affect antimicrobial susceptibility.

The limited number of relevant studies on CHO and BZT does not allow to conclude that the use of these actives could lead to increases in susceptibility to CHO or BZC, or/and chemotherapeutic antibiotics. There is no information on the impact of CHO or BZC on transfer or maintenance of resistant genes.

Despite the abundant literature on antimicrobial 'resistance', the practicality of the findings remains limited. The use of recognized standard for testing the activity of biocides and antibiotics would enable a better comparison between data and provide some clinical significance. The debate about how to use MIC data is not over since the determination of MIC is less time consuming and can be automated. The use of relevant test concentration such as the 'during use' concentration would enable to interpret better the practical significance of an increased MIC. The rise of isolate with multiple efflux genes carriage may be a concern, but the clinical significance of such carriage needs to be established. Likewise, the impact of biocides (generally) on maintenance of efflux genes (or others), forced mutations and gene transfer still required further investigations. At the end of the day, the use of biocides to control/eliminate pathogens remains essential in many environments, and this needs to be appropriately balanced realistic risks of emerging antimicrobial resistance.

AUTHOR CONTRIBUTION

Jean-Yves Maillard conducted the search for, read and analysed, all 3655 articles, wrote the entire review, design the tables and figures, and edited the manuscript.

CONFLICT OF INTEREST

The American Cleaning Institute commissioned Biocide Consult Ltd to write a comprehensive report on BKC, BZT and CHO resistance to help with a submission of a document on the impact of BKC, BZT and CHO on AMR to the US FDA. This review is based partly on that report. [Correction added on 21 October 2022, after first online publication: The American Clinical Institute has been corrected to The American Cleaning Institute in this version]

Jean-Yves Maillard is the Director of Biocide Consult ltd.

DATA AVAILABILITY STATEMENT

Supplementary tables on which this manuscript is based are available as supplementary materials and have been uploaded alongside the manuscript

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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