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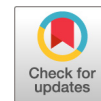
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# Benzalkonium Chlorides: Uses, Regulatory Status, and Microbial Resistance

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**ABSTRACT** Benzalkonium chlorides (BACs) are chemicals with widespread applications due to their broad-spectrum antimicrobial properties against bacteria, fungi, and viruses. This review provides an overview of the market for BACs, as well as regulatory measures and available data on safety, toxicity, and environmental contamination. We focus on the effect of frequent exposure of microbial communities to BACs and the potential for cross-resistant phenotypes to emerge. Toward this goal, we review BAC concentrations in consumer products, their correlation with the emergence of tolerance in microbial populations, and the associated risk potential. Our analysis suggests that the ubiquitous and frequent use of BACs in commercial products can generate selective environments that favor microbial phenotypes potentially cross-resistant to a variety of compounds. An analysis of benefits versus risks should be the guidepost for regulatory actions regarding compounds such as BACs.

**KEYWORDS** alkyl dimethyl benzyl ammonium chlorides, antiseptic, BACs, benzalkonium chlorides, QACs, resistance

## WIDESPREAD USE IN A MULTIBILLION-DOLLAR MARKET

**B**enzalkonium chlorides (BACs), also known as alkyl dimethyl benzyl ammonium chlorides, alkyl dimethyl (phenylmethyl) quaternary ammonium chlorides, ammonium alkyl dimethyl (phenylmethyl) chlorides, or ammonium alkyl dimethyl benzyl chlorides, are a class of quaternary ammonium compounds (QACs) (Fig. 1A). They are usually commercialized as a mixture of compounds with different lengths for the alkyl chain, ranging from C<sub>8</sub> to C<sub>18</sub>, with higher biocide activity for C<sub>12</sub> and C<sub>14</sub> derivatives (1).

BACs were reported for the first time in 1935 by Gerhard Domagk, gaining the market as zephiran chlorides, and were marketed as promising and superior disinfectant and antiseptics (2). In 1947, the first product containing BACs was registered with the Environmental Protection Agency (EPA) in the United States (3). Since then, they have been used in a wide variety of products, both prescription and over the counter. Applications range from domestic to agricultural, industrial, and clinical (Fig. 1B). Domestic applications include fabric softeners (4), personal hygiene and cosmetic products, such as shampoos, conditioners, and body lotions (5), as well as ophthalmic solutions and medications that use the nasal route of delivery (6). BACs are also among the most common active ingredients in disinfectants (4) used in residential, industrial (7), agricultural, and clinical settings. Additional registered uses for BACs in the United States include applications on indoor and outdoor surfaces (walls, floors, toilets, etc.), agricultural tools and vehicles, humidifiers, water storage tanks, products for use in residential and commercial pools, decorative ponds and fountains, water lines and systems, pulp and paper products, and wood preservation (3). The recommended or allowed concentrations of BACs in different products vary considerably according to the application (Table 1). With perhaps the exception of countries which adopted

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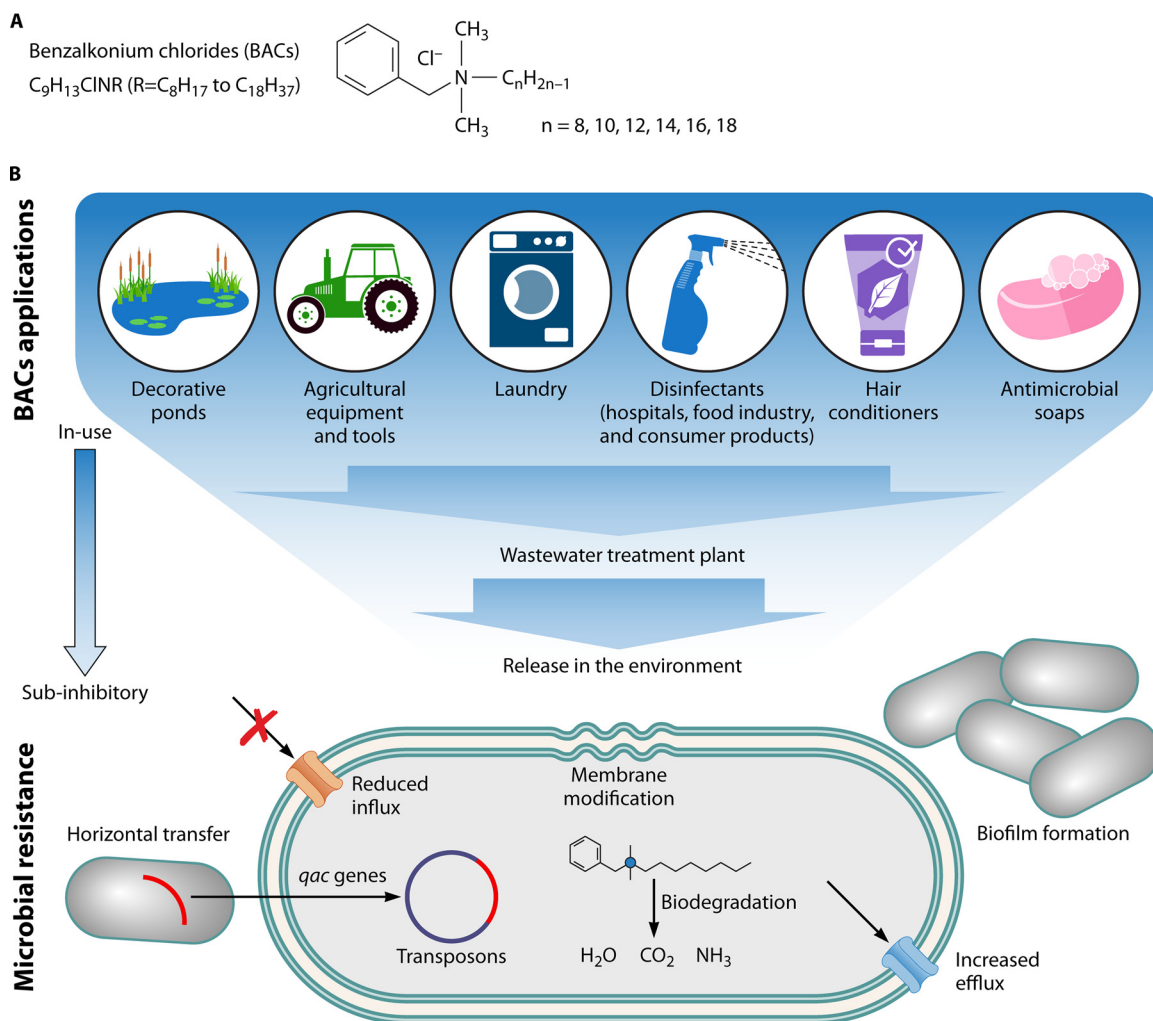
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**FIG 1** (A) Benzalkonium chloride (BAC) formula and structure. (B) Uses of BACs and six reported types of mechanisms of microbial resistance to BACs.

stricter regulations toward BAC use, discussed in the next section, the potential use of BACs is likely on the rise. The global market for disinfectants alone, which includes BACs, is expected to grow over 6% from 2016 and reach over \$8 billion by 2021 (8).

**CURRENT REGULATION**

In Europe, the European Commission (EC) is involved in the regulation of BACs. Recent rules in the European market included a change in the maximum residual levels of BACs allowed in food products from 0.5 mg/kg to 0.1 mg/kg, values which will undergo an additional review by the end of 2019 (9). Additionally, recent changes in legislation, Decision (EU) 2016/1950 and the Biocidal Products Regulation (EU) no. 528/2012 (the BPR) (10, 11), meant that BACs are no longer approved for use in several biocidal products, such as consumer hand and body wash antiseptics, which is in contrast with current legislation in the United States.

In the United States, the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) share the role of regulating BACs. Such agencies regularly update regulations based on current scientific data, occasionally limiting the use of compounds found not to be safe or effective. Final determinations, however, can be delayed by requests from the industry sector that commercializes such products. As an example, the FDA recently published three proposed and final decisions regarding the use of chemicals as consumer hand rub antiseptics, consumer hand and body wash

**TABLE 1** BAC concentrations found or allowed in commercial products, the environment, and substances to which microbes are resistant or tolerant and that cause toxic effects

Description <sup>a</sup>	Category <sup>b</sup>	Reported BAC concn <sup>c</sup>	Reference <sup>d</sup>
BAC-C <sub>12</sub> detected in wastewater treatment influents (maximum amt)	E	170 µg/liter	101
BACs detected in food samples (maximum amt)	E	14.4 mg/kg	38
Groundwater and reclaimed water	E	Up to 0.2 µg/liter	36
Wastewater samples	E	Up to 37 µg/liter	102
Wastewater sites that included hospital, laundry, dairy, swimming pools, paper production	E	Up to 2.8 mg/liter for BAC-C <sub>12</sub> , 1.1 mg/liter for BAC-C <sub>14</sub> , 0.027 mg/liter for BAC-C <sub>16</sub>	33
Effluents from European hospitals	E	0.05 to 6.03 mg/liter	34
Sediment samples from metropolitan region of the lower Hudson Basin	E	1.5 mg/kg	37
Wastewater treatment plant	E	Up to 0.17 mg/liter	32
Wastewater from hospital and laundry	E	2.8 and 2.1 mg/liter	32
Municipal sewage sludge in China	E	0.09–191 mg/kg	103
Ophthalmological solutions	P	0.003–0.02%	29
Hair conditioner	P	0.5–2%	5
Zephiran chloride recommended concn after dilution of 17% product concentrate	P	0.03–0.2%	104
Mosquitocide	P	200 ppm	3
Legislation limits for food processing plants, equipment, and utensils	P	200–400 ppm	3
Maximum concn that can be safely used in contact products, according to the American College of Toxicology	P	0.10%	23
Disinfectant (Lysol)	P	0.1% (wt/vol)	35
Maximum allowed wastewater for rinse-off hair products (Europe)	P	3%	105
Disinfectant in food-processing areas (Denmark) before 2016	P	3%	106
Preservative (Denmark) before 2016	P	0.10%	106
Biocidal preservative in masonry (France/UK) before 2016	P	<2.5%	106
Water preservative for cooling systems (Denmark) before 2016	P	9–50%	106
Chemical toilets before 2016	P	10–15%	106
Antimicrobial soaps and antiseptics (Hy-G-Clenz; Global Industrial Advanced; Neosporin wound cleanser for kids, cleanse antibacterial hand soap)	P	0.13%	NA
Maximum allowed BACs in food products in Europe regulation 1119/2014/EU	P	0.1 mg/kg	9
MIC of BACs for <i>L. monocytogenes</i> isolates from food-processing plants	R	7 mg/liter	50
<i>Pseudomonas</i> strain resistant to ammonium acetate-buffered BACs	R	0.40%	43
MIC of BACs for methicillin-resistant <i>Staphylococcus aureus</i> strains resistant to BACs	R	5–10 mg/liter	52
MIC of BACs for <i>Escherichia coli</i> K-12 resistant to BACs	R	92 mg/liter	53
MIC of BACs for <i>Salmonella</i> strain resistant to BACs	R	256 mg/liter	54
MIC of BACs for <i>E. coli</i> O157 resistant to BACs	R	1,024 mg/liter	54
MIC of BACs for <i>L. monocytogenes</i> in BACs	R	40 mg/liter	107
MIC of BACs for <i>E. coli</i> HB101 parent strains and strain overexpressing <i>mdfA</i>	R	50 and 400 mg/liter	72
MIC of BACs for <i>E. coli</i> KAM32 parent strain and strain overexpressing <i>pmpM</i>	R	1.2 and 37.5 mg/liter	74
<i>P. aeruginosa</i> (nonadapted and adapted) tolerance to BACs	R	1,200 and 1,600 mg/liter	56
MIC of BACs for <i>L. monocytogenes</i> wild-type and with <i>emrE</i> deletion	R	30 and 10 mg/liter	71
MIC of BACs for multiple isolates of <i>P. aeruginosa</i> strains	R	70–625 mg/liter	93
MIC of BACs for <i>E. coli</i> strains before and after adaptation to BACs, respectively	R	25 and 150 mg/liter	89
MIC of BACs for <i>Listeria</i> spp. with BAC resistance	R	35–40 mg/liter	77
MIC of BACs for isolates of carbapenem-resistant <i>Acinetobacter baumannii</i>	R	4–64 µg/liter	92
MIC of BACs for <i>P. aeruginosa</i>	R	0.45 mM	84
MIC of BACs for <i>E. coli</i>	R	0.225 mM	84
MIC of BACs for <i>L. monocytogenes</i> after adaptation	R	30 mg/liter	55
MIC of BACs for <i>Campylobacter coli</i> after adaptation for 15 days	R	2 mg/liter	46
MIC of BACs for <i>P. aeruginosa</i> after adaptation to BACs for 30 days	R	350 mg/liter	60
MBC of BACs for MRSA and MRSP isolates	R	2.1–135 mg/liter	108
Inhibition of growth for planktonic cells of <i>S. Typhimurium</i> , no effect over mature biofilms from the same strain	R	0.01%	80
Causes skin irritation	T	0.5–1%	20
Toxic effects to human conjunctival cells ( <i>in vitro</i> )	T	0.0001–0.1%	22
Algal growth inhibition	T	0.255 mg/liter	31

(Continued on next page)

TABLE 1 (Continued)

Description <sup>a</sup>	Category <sup>b</sup>	Reported BAC concn <sup>c</sup>	Reference <sup>d</sup>
NOAEC for aquatic invertebrates	T	4.15 $\mu$ g ai/liter	3
Chronic NOAEC for mammals	T	44 mg/kg/day	3
Chronic effects in fish	T	32.2 $\mu$ g ai/liter	3
Genotoxic effects in plant and mammalian cells ( <i>in vitro</i> )	T	1 mg/liter	21
Toxic to <i>Ceriodaphnia dubia</i> (crustacean)	T	0.004 mg/liter	30
Superficial cell loss observed in the cornea of rabbits with an ophthalmological solution containing BACs	T	0.02%	25

<sup>a</sup>MBC, minimal bactericidal concentration; MRSP, methicillin-resistant *Staphylococcus pseudintermedius*; NOAEC, no observable adverse effect concentration.

<sup>b</sup>P, commercial products; E, environment; R, substance to which microbes are resistant or tolerant; T, causes toxic effects.

<sup>c</sup>ai, active ingredient.

<sup>d</sup>NA, not applicable.

antiseptics, and health care antiseptics (12–14). The rules banned specific biocides, such as triclosan, or added additional and stricter regulatory approvals for several others, such as chlorhexidine, regarding the applications mentioned above. In all instances, however, BACs were excluded from the decisions and granted deferral letters as requested by manufacturers. The decisions granted manufacturers extra time to provide data to fill gaps related to safety and efficacy. Since 2015, letters and recommendations have been moving back and forth between the FDA and manufacturers and their representatives, such as the American Cleaning Institute, Lonza America, and Henkel Consumer Goods, Inc. (15–19). Decisions to postpone any action regarding the regulation of BACs were taken based on the affirmation of lack of sufficient data in the literature. Yet, multiple researchers have studied the safety aspects of BACs over the years, which include data on the toxicity to humans and the environment, as we discuss next.

## TOXICITY TO HUMANS

The toxicity of BACs to humans and other animals has been described in the literature, even though discordant conclusions arise from differences in experimental conditions. As reviewed elsewhere (20), BACs are known skin irritants, with occasional, rarer reports as allergens (skin sensitizers). Regarding acute toxicology data, BACs are classified by the EPA as toxicity category II by the oral and inhalation routes and toxicity category III via the dermal route (3). They are also considered to be highly irritating to the eyes and skin (toxicity category I) (3). Small but significant genotoxic effects in both plant and mammalian cells were observed *in vitro* for BAC concentrations as low as 1 mg/liter, which is lower than those reported to be found in the environment (21). Considerable cell toxicity was observed *in vitro* for human ocular cells exposed to BAC concentrations as low as 0.0001% (22).

In contrast, a few reports in the literature found BACs to be considered safe. A report from 2006 by the EPA did not recognize BACs as being carcinogenic, mutagenic, or genotoxic (3). Regarding their addition to intranasal products, a review of 18 studies from the literature revealed no major safety concerns when BACs were used in concentrations ranging from 0.00045% to 0.1% (23). A recent review of BAC safety in cosmetic products (5) regarded their use as possibly safe, based on calculations of the margin of safety (MOS), a formula which considered the concentration of BACs in products, use frequency, and amount, and estimated parameters such as no observed adverse effect level (NOAEL) and dermal absorption ratios.

For the specific application of ophthalmological solutions, a study sponsored by Alcon Laboratories concluded that there was no safety difference between those with or without the addition of BACs (24), even though multiple researchers reported pathological effects when ophthalmological solutions containing BACs as a preservative were used, compared to preservative-free solutions (25, 26). Multiple reports of BAC toxicity for such application have even motivated the development of preservative-free ocular solutions (27). Labeling recommendations from the European Commission for

medicinal products containing BACs have also recognized eye irritation as a toxic effect from BACs (28).

In summary, most studies and governmental agencies agree that BACs are not innocuous substances, even when used in small concentrations (3, 20–22, 25, 26, 28). Safety concerns regarding their use are frequently associated with long-term contact product use, such as in preservatives in medications used by glaucoma patients, which can be chronically exposed to BACs (22, 25, 26, 29).

## ENVIRONMENTAL CONTAMINATION

In a 2006 report, the EPA recognized the toxicity of BACs to the aquatic environment and its inhabitants, such as fish, oysters, shrimp, and invertebrates, advising against the release of BACs into lakes, oceans, or other waters (3). Since then, their toxicity to aquatic organisms, as well as other animals, has been well established by several research groups (30, 31). Despite that, BACs have been detected in wastewater effluents and other environments (Table 1).

Data regarding the detection of BACs in the environment are sparse in the literature, and recent measurements are lacking. BACs were reported in wastewater effluents from hospitals, reaching concentrations in the milligram-per-liter range (32–34). Other effluents, such as those from laundry, dairy, community pools, also had the presence of BACs (32, 33) at various concentrations that were generally lower than those originating from hospitals. Typical wastewater treatment plants are not designed to treat QAC contaminants, resulting in the release of at least a portion of them into the environment as micropollutants (35). Concentrations varying in the ranges of microgram per liter or microgram per gram were found in ground and reclaimed water (36), as well as soil samples (37). BACs were also detected in up to 3.5% of over 4,000 food samples analyzed by the European Food Safety Authority (EFSA) (38).

From all the QACs tested by different research groups, BACs, mainly  $C_{12}$ ,  $C_{14}$ , and  $C_{16}$ , were found in higher concentrations than in other QACs (32, 33). The high incidence of BACs could be attributed, at least in part, to their popularity in various applications, from consumer products such as eye drops, shampoos, and mosquito insecticides, to disinfectants and antiseptics used in hospitals and food industries. Whether the widespread use of these compounds, a lack of proper disposal, or a combination of both contributed to the observed incidence in the environment is unknown. We estimate that BAC disposal in the environment is still considerable, especially in countries that have less-restrictive legislation, such as the United States. Deeper investigations, however, are required to establish the current levels of BACs in the environment, as well as the potential links for the development of resistant microbial strains, which we discuss next.

## MICROBIAL TOLERANCE AND RESISTANCE

The use of BACs for multiple applications, many of which unavoidably result in the generation and release of residual biocide, can result in the presence of environments in which there is a selection pressure over microbes to evolve resistance to such chemicals (35). The capacity of bacteria to survive and thrive in BACs has been demonstrated by tracking outbreaks usually associated with misuse or improper dilution and storage of disinfectants and antiseptic solutions (39). In fact, multiple outbreaks were associated with BACs throughout 4 decades (39), motivating a series of recommendations to discontinue their use as an antiseptic (40, 41).

Concerns about the use of BACs as antiseptics are not novel, and researchers have observed resistant strains capable of surviving in BAC solutions (0.1 to 0.4%) as early as the 1960s (42, 43). It is known that bacteria can adapt and increase their tolerance to stressful chemicals (44, 45), and such phenomena have been shown repeatedly for BACs. Frequently, the adaptive mutations that select for increased tolerance or resistance are stable at a population level and can be still observed for evolved strains even after the selection pressure has been lifted (46). Even though the reported concentrations vary depending on the study and the bacterial genera (Table 1), it has been

demonstrated that bacteria can evolve to survive to BAC concentrations similar to those found in the environment and in consumer products (Table 1).

It is important to highlight that the terms “tolerance” and “resistance” have been used interchangeably, especially when related to biocides, which could lead to misinterpretation of data (47, 48). Resistance is broadly understood as the “insusceptibility of a microorganism to a particular treatment under a particular set of conditions” (47, 48). Multiple researchers defined resistance based solely on an increase in the MIC (49, 50). The term tolerance has been used on several distinct occasions. Tolerant strains were defined as those in which the antimicrobial’s MIC for them did not increase, but the strain was able to survive killing by, for example, reducing growth (51). Tolerant strains were also defined as those in which the antimicrobial’s MIC for them increased compared to the controls (48). We believe that the broad term “decrease in susceptibility” is often more appropriate to describe the observed increases in MIC for biocides, including the following examples.

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were evolved in BACs (52), doubling the MIC of BACs from 5 to 10 mg/liter, after a period of adaptation. The MICs of BACs increased 4-fold for *Campylobacter coli* after exposure to the chemical for 15 days (46). *Escherichia coli* K-12 strains exposed to increasing concentrations of BACs were able to survive in a concentration of 92 mg/liter BACs, which was eight times higher than the concentration in which the parent strain could survive (53). Another study showed that the MICs of BACs changed from 4 to 256 mg/liter for *Salmonella enterica* serovar Virchow and reached over 1,000 mg/liter for *E. coli* O157 (54). The leading foodborne pathogen in the United States, *Listeria monocytogenes*, is also capable of decreasing its susceptibility to BACs. Three different strains (H7550, SK2802, and J0161) of *L. monocytogenes* from outbreaks and disease cases were exposed to BACs, and isolates with up to 3-fold increases (10 to 30 mg/liter) in the MICs of BACs were obtained for all strains (55).

The *Pseudomonas* sp. strains can naturally withstand the highest concentrations of BACs. *Pseudomonas aeruginosa* survives at up to 1,600 and 1,200 mg/liter BACs with or without a previous adaptation to the chemical, respectively (56). The MIC of BACs for the isolated strain *Pseudomonas* sp. BIOMIG1 was 1,024 mg/liter (57). The higher recalcitrance of *Pseudomonas* spp. may explain why, after exposing complex microbial communities to BACs, there was an enrichment in *Pseudomonas* species, with a decrease in microbial diversity (58, 59). In another study, *P. aeruginosa* NCIMB 10421 was cultivated in continuous culture, and the BAC concentration was progressively increased for about 30 days. The MICs of BACs increased from 25 mg/liter to over 350 mg/liter, and the adapted strain had higher fitness when competed with the parent strain in the presence of BACs, especially with magnesium depletion and the presence of glucose in the medium (60).

A recent study has questioned the use of aqueous solutions of BACs to determine their activity against microorganisms, demonstrating that BACs in real-use formulations (with surfactants and chelating agents) is more effective to control microbial growth (59). Despite this finding, strains with decreased susceptibility to BACs can not only develop and be selected for under controlled laboratory conditions, but they have also been isolated directly from real-case scenarios, environments in which BACs is frequently used as a biocide. Strains of the pathogen *L. monocytogenes* isolated from diverse environments, such as food-processing plants, food products, patients, and animals, were reported as having decreased susceptibility to BACs. Such strains ranged from 8% (49) and 10% (50) up to 40% (61) and 45% (62) of the total number of isolates in these environments.

### MICROBIAL MECHANISMS OF TOLERANCE AND RESISTANCE

The mode of action of QACs, including BACs, involves the perturbation and disruption of the membrane bilayers by the alkyl chains and disruption of charge distribution of the membrane by the charged nitrogen (63). Accordingly, susceptibility to BACs may emerge through a combination of mechanisms (56), with many of those related to the

cell membrane. The mechanisms proposed in the literature include changes in the overall membrane composition, downregulation of porins, overexpression or modification of efflux pumps, horizontal gene transfer of transposon elements and stress factors, biofilm formation, and biodegradation (Fig. 1B).

Changes in the membrane composition have long been associated with decreased susceptibility to BACs (64, 65). Resistant strains of *P. aeruginosa* were shown to have different phospholipid and fatty acid compositions compared to a susceptible strain (64). Other work has demonstrated that exposure of *Bacillus cereus* to BACs induced genes involved in fatty acid metabolism and caused changes in the fatty acid composition of the membrane (66). The authors, however, did not evaluate whether exposed strains exhibited a tolerant phenotype. A strain of *E. coli* with reduced susceptibility to BACs was shown to have a lipopolysaccharide composition diverse from that of the susceptible strain (64). Recently, it was suggested that *Pseudomonas* strains could partially adapt to BACs by stabilizing the membrane charge through the increase in polyamine synthesis gene expression and mutations in *pmrB* (56).

The reduced influx of BACs has been suggested to collaborate to decreased susceptibility to the biocide. Since adsorption of QACs is believed to occur through porins (63), decreased susceptibility could be achieved, in theory, by the downregulation of porins. In accordance, the downregulation of genes for multiple porins has been associated with *Pseudomonas* (56, 67) and *E. coli* (53) strains less susceptible to BACs. A lower level of the porin OmpF in the *E. coli* membrane decreased the strain susceptibility to BACs (64). A causal relationship between a disinfectant product containing BACs and the downregulation of porins was demonstrated for *Mycobacterium smegmatis*; knockout mutants for Msp porins were less susceptible to the biocide than was the wild type (68). The use of a disinfectant formulation by the authors, however, limits the extent to which the observed effect can be attributed to BACs, other components of the formula, or the mixture. Further studies are required to reinforce the link between tolerance to BACs and the downregulation of porins.

The presence or upregulation of certain families of efflux pumps has been associated with multidrug resistance and decreased susceptibility to BACs across several genera of bacteria. Resistance via increased efflux lowers the concentration of biocide inside the cell, allowing the bacteria to survive against higher environmental concentrations of the chemical. One such case is the Qac proteins, a group of multidrug efflux proteins frequently associated with resistance to BACs (69). In the foodborne pathogen *L. monocytogenes*, the efflux pump MrdI (70) and the efflux pump EmrE (71) have been associated with resistance to BACs. In isolates of *L. monocytogenes*, the susceptibility to BACs and antimicrobials could be restored when the efflux inhibitor was added to the medium containing a previously adapted and resistant strain. This suggested at least a partial role of efflux pumps for resistance to BACs in this organism (55). The efflux protein MdfA contributed to increased resistance to BACs in *E. coli* (72). For the plant pathogen *Pseudomonas syringae*, the resistance-nodulation-division (RND)-type pump MexAB-OprM knockout mutant showed increased sensitivity to BACs (73). Another efflux pump, the PmpM of the multidrug and toxin extrusion (MATE) family, from *P. aeruginosa*, contributed to decreased susceptibility to BACs when expressed in a plasmid in *E. coli* (74). Accordingly, the exposure of *Pseudomonas* strains to BACs for a long time resulted in the overexpression of multidrug efflux pump genes (56). Mutations in the *nfxB*, a regulator for the Mex efflux system, as well as overexpression of both MexAB-OprM and MexCD-OprJ efflux systems and downregulation of *mexR*, a repressor of the Mex system, was also correlated to decreased sensitivity to BACs in *P. aeruginosa* (60).

Resistance elements, like efflux pumps, often appear to be associated with other genes, such as mobile elements and transposases (75), which contributes to their dissemination in bacterial populations and maintenance of tolerant and resistant phenotypes. The transposon Tn6188 was associated with strains of *L. monocytogenes* with increased tolerance to BACs. It included three transposases and a protein which was similar to the Smr, EmrE, and Qac efflux proteins (75). Strains of *L. monocytogenes*



responsible for outbreaks in Canada had a genomic island containing multiple resistance, stress response, and virulence-associated genes (76), which included an efflux pump involved in resistance to BACs (71). Successful horizontal gene transfer of resistance-associated genes from nonpathogenic BAC-resistant *Listeria innocua* and *Listeria welshimeri* to the pathogenic *L. monocytogenes* does occur, and it suggests that more common nonpathogenic strains frequently exposed to the biocide in food-processing plants can act as resistance reservoirs (77).

Factors such as the presence of biofilms can affect the ability of a biocide to control and eliminate microorganisms (78). Biofilms are communities of single- or multispecies microorganisms attached to solid surfaces surrounded by their secreted exopolysaccharide matrix. Biofilm formation represents one of the mechanisms of resistance and tolerance explored by bacteria to avoid and protect themselves against stressful environments (79). Bacterial communities in biofilms have increased ability to survive antiseptics and disinfectants, such as BACs, compared to planktonic cells (80). Exposure of *Salmonella enterica* to 0.02% of BACs (2-fold higher than the MIC for planktonic cells) for between 10 and 90 min, though it reduced the cell number, failed to eradicate the biofilm (79).

Tolerance to BACs can be greater for multispecies biofilms than for single-species biofilms, as was the case for a dual-species biofilm with *L. monocytogenes* and *Pseudomonas putida* (78, 81). This result can be partially explained by the selection pressure for the strain with higher intrinsic resistance to the biocide (78). As mentioned before, *Pseudomonas* species naturally have a better capacity to survive in higher concentrations of BACs (56, 57). Their presence in the biofilm community could contribute to the increased tolerance compared to other single-species biofilms.

The cases mentioned above demonstrate the better capacity of both single cells and multispecies cells to survive the presence of biocides when in biofilms versus planktonic cells. In addition, the exposure to the biocide can occasionally increase biofilm formation by bacteria (82–84). Continuous exposure of bacteria to BACs resulted in thicker biofilms, as observed with scanning electron microscopy (SEM) (84). Strains of *E. coli* isolated from the dairy industry which were less susceptible to BACs and antibiotics also had an increased ability to form biofilms (82). The susceptible strains became strong biofilm formers as well after a period of adaptation (exposure) to BACs (82). Exposure to BACs induced biofilm formation by *Staphylococcus epidermidis* CIP53124, although the same effect was not observed for other species tested (83).

Last, some microbial communities and species such as *Pseudomonas* spp. are capable of degrading BACs, converting them into less toxic chemicals and utilizing them as secondary substrates and energy sources (58, 85). Degradation of BACs by dealkylation decreases its toxicity to microorganisms (86). A study of microbial communities suggested that *Pseudomonas* sp. strain BIOMIG1 was responsible for the biodegradation of BACs, possibly via dioxygenase (57). Degradation of BACs under nitrate-reducing conditions in the presence of a methanogenic culture obtained from an anaerobic digester has also been demonstrated (87). The transformation was determined to be abiotic by a nucleophilic substitution with nitrite that generated benzonitrile (87).

Given the mode of action of BACs through membrane disruption (63) and the above-described general mechanisms of bacterial response by membrane modification (64, 65), overexpression of multidrug efflux pumps (56, 70–74), and biofilm formation (78, 79, 81), we expect some level of cross-resistance to other antimicrobials, which is described next.

### CROSS-RESISTANCE TO ANTIBIOTICS

Cross-resistance is the phenomenon in which exposure to one chemical grants an advantage for survival in a distinct chemical (44, 45). Cross-resistance between antiseptics, disinfectants, and antibiotics has been thoroughly described in the literature, including cases involving BACs.

The antibiotics oxacillin, ceftazolin, and ofloxacin had higher MICs in methicillin-

resistant *S. aureus* (MRSA) strains evolved in the presence of BACs (52). MRSA strains nonadapted to BACs were already resistant to ofloxacin, as defined by EUCAST standards (88), and the MICs of the antibiotic increased up to 4-fold for the adapted strains (52). Similar results were observed with *E. coli* (53, 54, 89). The laboratory strain *E. coli* K-12 adapted to increasing concentrations of BACs. This resulted in several antibiotics, such as ampicillin, ciprofloxacin, and nalidixic acid, increasing the MIC on such a strain (53). MICs for multiple antibiotics also increased after adaptation to BACs for the pathogen strain *E. coli* O157 (54), and the same was observed for *E. coli* ATCC 11775 and DSM 682 (89). In some cases, *E. coli* strains adapted to BACs became resistant to antibiotics, as defined by EUCAST (88), such as chloramphenicol (54, 89) and ampicillin (89). The MICs of multiple antibiotics also increased after adaptation to BACs for the bacteria of *Salmonella* serovar Virchow (54). Such strains became resistant to amoxicillin, as defined by EUCAST (88), after exposure to BACs. *L. monocytogenes* strains adapted to BACs showed decreased sensitivities to both ciprofloxacin and gentamicin (55). *P. aeruginosa* evolved in the presence of BACs in continuous culture, on the other hand, exhibited varied sensitivities to antibiotics. The adapted strain PA-29 was less sensitive to ciprofloxacin but more sensitive to minocycline, which is an antibiotic similar to tetracycline (60). The authors believed that the increased sensitivity to minocycline was due to a decrease in the expression of the MexXY-OprM efflux pump system observed for the adapted strain (60), which plays a role in the resistance to an analogue of minocycline (90). They did not confirm this hypothesis, however.

Besides isolated strains, evidence of cross-resistance between BACs and antibiotics has been shown for microbial communities. The exposure of complex microbial communities to BACs not only decreased the overall diversity of the population but also resulted in decreased susceptibility to three clinically relevant antibiotics, penicillin, tetracycline, and ciprofloxacin (58).

Evidence of cross-resistance between BACs and antibiotics is not exclusively limited to controlled laboratory experiments and strains. Following the isolation of *S. aureus* strains from patients, the MIC of BACs increased for over 100 isolates, which corresponded to approximately half of the isolates. BAC-resistant isolates harboring plasmids with *qacA* and *qacB* genes were also less sensitive to multiple antibiotics than were BAC-sensitive ones. The incidence of *qac* and  $\beta$ -lactamase *bla* genes in the same plasmids provided strong evidence of a linkage between the selection pressure for resistance to disinfectants, such as BACs, and antibiotics, such as penicillin (91). A similar association occurred for over 50 isolates of carbapenem-resistant *Acinetobacter baumannii*. Strains obtained from four different hospitals had a high prevalence of both *qac* and *bla* genes (92).

In contrast, a study conducted by the Unilever group (93) questioned the correlation between biocide use and cross-resistance to antibiotics for *P. aeruginosa*. Their statistical analysis revealed a stronger link between biocides and antibiotic susceptibility between strains isolated from clinical settings than from industrial settings, which made the authors conclude that misuse of antibiotics, and not disinfectants, were driving the results. Though interesting, additional studies would be necessary to demonstrate such a conclusion. It is also not clear whether such a correlation would hold for other bacterial species.

The exposure and adaptation to BACs can result in decreased susceptibility to several clinically relevant antibiotics in some species (52, 54, 55, 58, 89, 91), but not all, and several studies have also reported the opposite result, i.e., increased susceptibility to antibiotics (60, 94, 95). Most studies do not report whether the observed increases in MIC for the antibiotics are within the definition of resistance according to clinical standards (88, 96). Such a fact often motivates questioning of the relevance of such studies (48, 93). However, an increase in MIC by itself demonstrates the existence of a cross-resistance effect and should not be ignored. Researchers showed that bacteria that are merely tolerant to antibiotics can develop resistance to them faster (51). The ability of bacteria to survive the presence of the antibiotics, even before the MIC has

reached clinical standards, helps keep and accumulate mutations that can eventually result in the emergence of strains resistant to the antibiotics (51).

## CONCLUSION

This review of the literature explored the data currently available on the potential implications of BACs to human safety and the environment in general. There is evidence that the continuous use of biocides and their release to the environment in subinhibitory concentrations may lead to the emergence of tolerant, resistant, and cross-resistant microbial strains, even though there are occasional controversial reports in the literature. Given the reported side effects of BACs, we believe that a thorough analysis of benefits versus risks should be the guidepost for future regulatory and manufacturing use of the compound. Based on the analysis presented here, we have a few recommendations.

We propose restrictions for BAC use in consumer products. Currently, the Centers for Disease Control and Prevention (CDC) recommends (97), and the FDA endorses (98), the use of only water and plain soap by regular consumers (which does not include professionals in health care settings). Despite that, BACs are still commercialized in over-the-counter antimicrobial soaps in the United States. The FDA has recently regulated other chemicals, such as triclosan and chlorhexidine, postponing any decisions regarding the use of BACs (12, 13).

Additionally, updated data regarding the presence of BACs in the environment, water, and soil are required to determine the need for monitoring such a compound and establishing a baseline of its concentration in various environments. Based on the available data, bacteria can survive BAC concentrations found in the environment (Table 1), and cross-resistance between BACs and antibiotics has been reported (52–55, 58, 89, 91).

Finally, we urge further research on the effect of BAC exposure, both in free form and as part of consumer products, to microbial populations and tissues to elucidate its toxigenic and long-term potential to alter the microbial flora in both a clinical and environmental context. We still have a limited understanding of the mechanistic underpinnings and basis of adaptation and how these link to the emergence of global health challenges like antibiotic resistance. Another link that remains to be determined is the impact of BACs and QACs in general to the human microbiota of the skin, gut, and others, which are lately associated with numerous diseases and performance outcomes (99, 100).

Balancing the concentrations that effectively inhibit bacteria in products, are not toxic to users, and will not leave residual pollutants after disposal is certainly challenging. Limiting the use and regulating and monitoring chemicals such as BACs are important to reduce the negative impacts on humans and the environment.

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