

General Interest

Relationship of Sanitizers, Disinfectants, and Cleaning Agents with Antimicrobial Resistance

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ABSTRACT

Sanitizers, disinfectants, and cleaning agents are vital to food hygiene assurance and are a major public health protection measure. Limiting microbial antibiotic resistance is also a global public health priority. Although many factors contribute to the rise in antimicrobial resistance in bacteria infecting humans, antibiotic use in both human clinical settings and for food-producing animals are primary contributors. Some concerns have been raised about the possibility of coselection between food hygiene chemicals and reduced antimicrobial susceptibility. This article reviews available evidence from individual studies purporting to demonstrate a possible risk of antimicrobial resistance development, following biocide usage. Furthermore, the conclusions of several key expert reports and meta-analysis publications were assessed for supportive evidence of a relationship between biocide usage in food production and resistance development. Although many studies report on the isolation of antimicrobial-resistant bacterial strains in food, evidence is lacking on the attribution of this resistance to biocide usage. Also, although a theoretical risk of causality exists, many of the studies performed to demonstrate this are *in vitro* studies using laboratory-grown or -trained bacterial isolates, challenged with sublethal (below the recommended food industry) disinfectant or sanitizing agent concentrations. The proper use of, and adherence to biocide manufacturer's instruction for use, and the avoidance of biocide active agent dilution (e.g., through biofilm presence) must be ensured in food production environments. It is recommended that *in situ* studies should be performed to further assess causality, ensured a clear differentiation between interpretation of stable antimicrobial resistance and phenotypic adaptation. Furthermore, authorization of new biocidal active substances should take a scientific and risk-based approach regarding the potential for driving microbial resistance.

HIGHLIGHTS

- Sanitizers and disinfectants (biocides) are essential for food safety assurance.
- Concerns have been raised about theoretical risk of biocide-induced antimicrobial resistance.
- *In vitro* studies provide weak causal evidence to attribute antimicrobial resistance to biocide usage.
- GMPs, proper biocide usage, and avoidance of biofilms mitigate risk of antimicrobial resistance.

Key words: Biocides; Coselection; Cross-resistance; Disinfection; Food hygiene; Microbial resistance

Antimicrobial resistance (AMR) has become a 21st century global public health threat and limiting its further emergence and spread has become a worldwide public health priority. Since 2015, the global action plan on AMR has been endorsed by the tripartite of Food and Agriculture Organization, World Organization for Animal Health, and World Health Organization (49), advocating the prudent use of antimicrobial compounds, preventing their unnecessary use, and including the phasing out of antimicrobial agents as

growth promoters (in food-producing animals). Regulatory focus internationally is on limiting the potential for AMR, given the importance of these drugs to human health. The European Union-wide ban on antibiotics' use as growth promoters in animal feed has been in place since January 2006 (2). In the United States, the use of subtherapeutic doses of antibiotics in animal feed and/or water to promote growth and improve feed efficiency was prohibited as of October 2015 by the U.S. Food and Drug Administration Veterinary Feed Directive (42). An ad hoc CODEX Intergovernmental Task Force on Antimicrobial Resistance has begun work to develop science-based guidance on the

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management of foodborne AMR, in line with the World Health Organization “Global Action Plan on Antimicrobial Resistance” and the “One Health” approach (16).

It is generally accepted that the misuse, overuse, or abuse of antimicrobial drugs in clinical, livestock or aquaculture, or for pet treatment contributes substantially to the development of antibiotic-resistant bacteria (25). It has been estimated, on the basis of Food and Agriculture Organization data, that 80% of antimicrobial use is in agriculture for food-producing animals. Van Boeckel et al. (44) reported that over two-thirds of projected future use of antimicrobial agents would be for animal or aquaculture production. Therefore, there has been a clear emphasis on the prudent use of antibiotics in agriculture, especially for those antibiotics considered important for clinical use.

Multiple sources of environmental contamination by antimicrobial residues or antibiotic-resistant bacteria or both have been documented, including waste or wastewater from pharmaceutical, hospital, and water treatment facilities (17, 25). Soil amendments (manures) and irrigation water have also been reported as potential ecological sources and means of transmission for AMR (17).

Some concern has also been raised regarding the use of metal-based bactericides in plant and animal production, leading to the possibility of selection of antimicrobial-resistant bacteria, through the process of coresistance and cross-resistance with certain metal ions (17, 31, 50). Similarly, the role of biocides or sanitizing agents in food production and manufacturing facilities, as well as in hospital settings, has been linked to the risk of increasing antibiotic resistance (9, 11, 17, 37), through similar selective pressures. In the European Union, authorization for the use of active substances and biocidal products includes the requirement for satisfactory assessment of resistance, cross-resistance, or adaptation to these same compounds (13). The U.S. Environmental Protection Agency does not currently require a company registering a biocidal product for hygienic, food, or health care use to assess the resistance, cross-resistance, or adaptation to the active ingredient or final product.

Biocides or sanitizing agents play a crucial role in various stages of the food production chain. They are widely used for cleaning and disinfection of areas associated with livestock and produce production, including farm buildings, equipment, and transportation vehicles, as well as being used directly on animals (e.g., in footbaths or for udder cleansing) (33, 37). Biocides are also extensively used in food manufacturing, including disinfection of the factory and retail equipment, containers, and environments. Their use, as demonstrated over centuries, ensures the hygiene status of food processing environments, controlling microbial pathogens and spoilage microorganisms (22). For example, the U.S. Interagency Retail *Listeria monocytogenes* Risk Assessment (43) estimated that the predicted risk of listeriosis from the consumption of ready-to-eat products sliced or prepared in retail deli departments increases by approximately 41% if wiping, washing, and sanitizing activities are not performed.

Although biocides are critical in the armory for food safety and hygiene, any evidence that their proper or

improper use could contribute to the emergence of bacteria with an antibiotic-resistant phenotype, should not be ignored. This article examines recent evidence for the coselection of AMR in bacteria exposed to commonly used biocides or sanitizing agents in food-manufacturing facilities.

TERMINOLOGY AND DEFINITIONS USED IN CONTEXT OF AMR AND BIOCIDES

Antibiotics are drugs of natural or synthetic origin that have the capacity to kill or to inhibit the growth of microorganisms (15). Antimicrobial agents are any substance of natural, semisynthetic, or synthetic origin that at in vivo concentrations kills or inhibits the growth of microorganisms by interacting with a specific target. The term antimicrobial is a collective for antiviral, antibacterial, antifungal, antiparasitic, and antiprotozoal agents (8). Although antibiotics are technically a subset of antimicrobial agents, the two terms are used interchangeably in this article and in the context of resistance.

Disinfectants, sanitizing agents, and cleaning chemical agents are used to inhibit growth or kill microorganisms to render them to levels that are safe in a food manufacturing facility. Biocides is an all-encompassing term for these chemicals, and their subclassification into specific categories, such as sanitizers and disinfectants, is mostly defined by the practical usage of these agents (46). They may be composed of specific formulations containing single or multiple active biocidal agents that may indiscriminately target bacterial cell structures. Active agents in commercially available biocides used in the food industry include quaternary ammonium compounds (QACs), benzalkonium chloride (BAC), chlorohexidine, chlorine and chlorine-based derivatives, acid anionic agents, hydrogen peroxide, and peracetic acid.

In general, AMR is the capacity of a microorganism to resist the growth-inhibiting or killing activity of an antimicrobial agent, beyond the normal susceptibility of the bacterial species (1, 27). This resistance can be intrinsic or acquired. Acquired resistance is the genetic variation or altered gene expression within certain strains of a microbial species, resulting in them differing significantly in susceptibility to a selection pressure. On the other hand, intrinsic resistance is the innate greater resistance exhibited by some microbial species or strains against certain antimicrobials, and manifested either as reduced susceptibility or survival against what will otherwise be a terminal challenge without any prior long-term exposure (46).

For antibiotic resistance, a clear distinction is also made between microbiological resistance (in vitro resistance) and clinical resistance (in vivo resistance) (46). The former is defined by the presence of an acquired or mutational resistance mechanism to the specific agent in comparison to a fully susceptible “wild type” (46). Clinical resistance defines when phenotypic testing of a microorganism-antibiotic combination against a clinical break point indicates that the concerned bacterium cannot be treated and therapeutic failure is inevitable (45).

A key parameter of an antibiotic-microbe interaction is the MIC, indicating a concentration of an antibiotic that

prevents growth of the microorganism and therefore likely to be therapeutically effective. Although many studies referring to biocide resistance are conducted by determining resultant changes in the MIC, other studies conduct MBC tests, which is defined as the lowest concentration of a biocidal agent required to kill (e.g., a 5-log reduction) a particular bacterium, over a fixed period under a specific set of conditions. This MBC can be considered complementary to the MIC: MIC test demonstrates the lowest level of antimicrobial agent that inhibits growth; the MBC demonstrates the lowest level of an agent resulting in microbial death. Therefore, although increases in MICs related to antibiotic susceptibility are useful indicators of decreased susceptibility or increased resistance, we should exercise care with similar interpretations for biocide susceptibility (46).

Often terms such as increased resistance, reduced tolerance, decreased susceptibility, and increased MIC are used interchangeably among studies to describe the altered response of bacteria to antimicrobial or antibiotic agents. In some cases, increased microbial resistance has been described as synonymous with reduced susceptibility and increased MIC, yet the targeted microorganism may revert to a wild-type phenotype when the selective pressure is removed. For the purpose of this review of evidence, an attempt is made to differentiate resistance and reversible habituation (phenotype adaptation): processes that are neither inherited nor transferable nor sustained after the selection pressure is removed. The terms “stable resistance” versus “transient resistance” have been introduced previously (28); furthermore, Kastbjerg and Gram (23) indicated that biocides could lead to an increased tolerance (i.e., survival without expressing resistant mechanisms or actual resistance). They described resistance as a sustained severalfold change in MIC. Coresistance is a generic term describing the selection of multiple antibiotic resistance genes when one of these genes is selected, often used to describe the linked changes in susceptibility between different agents, such as antibiotics, biocides, and heavy metals. Coresistance arises from the joint transfer of genetic determinants (e.g., integrons, transposons, plasmids, and bacteriophage), encoding for distinct resistance mechanisms. Cross-resistance indicates resistance to unrelated agents (i.e., biocides and antibiotics) but with mechanisms of action that are related or overlap. Cross-resistance and coresistance may be clearly designated as coselection mechanisms (6, 17, 46).

Cleaning is defined as the removal of soil, food residue, dirt, grease, or other objectionable matter with cleaning agents, defined as products used to clean, and disinfectants or sanitizers, defined as items used to reduce bacteria to a level that does not compromise food safety or suitability (7).

MODES OF ACTIONS: BIOCIDES VERSUS ANTIMICROBIALS

Antimicrobial agents, sanitizers, and disinfectants have very different modes of action. The former interacts very specifically with unique structures or metabolic processes of the microbial cell, such as the ribosomes, specific bacterial enzymes, or with bacterial cell wall

synthesis. Antimicrobials are often used for therapeutic treatments at levels close to MICs to exert bacteriostatic or bactericidal effect *in vivo*, without causing any adverse toxic effects and usually in concurrence with the host immune system. Table 1 describes the mode of action of the major classes of antimicrobials and their associated resistance mechanisms.

In contrast to the antimicrobials, sanitizers and disinfectants interact nonspecifically and usually on multiple microbial targets, often causing lethal damage to biological membranes (e.g., QACs) or reacting nonspecifically with functional groups of proteins (e.g., peroxygen compounds), or the genetic material (e.g., aldehydes). These agents are invariably used at concentrations that are severalfold higher than the MICs, as they are intended to deliver multiple log reductions of target organisms present in diverse niches (e.g., dry surfaces in presence of organic load) often in a single application. The nonspecific nature and multiple mechanism of actions of these agents significantly reduce the chances of microorganisms to become resistant to these chemicals, especially at recommended in-use concentrations for food hygiene applications, mainly because it is less likely for multiple resistance mechanisms to develop in parallel that can confer complete resistance against a biocide (17, 46). Figure 1 illustrates various modes of action of chemical active substances used in sanitizers and disinfectants.

SELECTION FOR AMR BY EXPOSURE TO BIOCIDES

In theory, resistance to antimicrobials through exposure to biocides should be less likely to occur due to different modes of actions and applications. However, should biocide usage coselect for AMR, it can potentially occur either

1. through cross-resistance in which there is a shared mechanism of action between these two classes of chemicals, or
2. due to coresistance when gene(s) conferring reduced susceptibility to a biocide is selected along with antimicrobial-resistant gene(s).

A number of research studies have been reported in this area, and a summary of selected publications is presented in Table 2 and discussed in the following sections of the article, along with our commentary on the evidence provided by these studies. In summary and as discussed later:

1. many of the reports are association studies that do not prove causality between biocide exposure and AMR, and
2. studies that investigated causality have mostly used laboratory-adapted strains to sublethal concentrations of biocides, which is against the recommended best practice for the use of these agents in real-life conditions.

The findings also show that AMR due to sublethal biocide exposure can be varied (i.e., it can increase or decrease, depending on the combinations of antimicrobial agent or biocide tested), resistance can be either transient (conferred through, e.g., physiological adaptations) or

TABLE 1. Mechanisms of resistance against different antimicrobial classes^a

Antimicrobial class	Mechanism of resistance	Specific means to achieve resistance	Example(s)
Beta-lactams			
Examples: penicillin, ampicillin, mezlocillin, piperacillin, cefazolin, cefotaxime, ceftazidime, aztreonam, and imipenem	Enzymatic destruction	Destruction of beta-lactam rings by β -lactamase enzymes; with the beta-lactam ring destroyed, the antibiotic will no longer have the ability to bind to PBP and interfere with cell wall synthesis	Resistance of staphylococci to penicillin Resistance of <i>Enterobacteriaceae</i> to penicillins, cephalosporins, and aztreonam
	Altered target	Changes in PBPs; mutational changes in original PBPs or acquisition of different PBPs will lead to inability of the antibiotic to bind to the PBP and inhibit cell wall synthesis	Resistance of staphylococci to methicillin and oxacillin
	Decreased uptake	Porin channel formation is decreased (where beta-lactams cross the outer membrane to reach the PBP of gram-negative bacteria), so a change in the number or character of these channels can reduce beta-lactam uptake	Resistance of <i>Enterobacter aerogenes</i> , <i>Klebsiella pneumoniae</i> , and <i>Pseudomonas aeruginosa</i> to imipenem
Glycopeptides			
Example: vancomycin	Altered target	Alteration in the molecular structure of cell wall precursor components decreases binding of vancomycin so that cell wall synthesis is able to continue	Resistance of enterococci to vancomycin
Aminoglycosides			
Examples: gentamicin, tobramycin, amikacin, netilmicin, streptomycin, and kanamycin	Enzymatic modification	Modifying enzymes alter various sites on the aminoglycoside molecule so that the ability of this drug to bind the ribosome and halt protein synthesis is greatly diminished or lost entirely	Resistance of many gram-positive and gram-negative bacteria to aminoglycosides
	Decreased uptake	Change in number or character of porin channels (through which aminoglycosides cross the outer membrane to reach the ribosomes of gram-negative bacteria) so that aminoglycoside uptake is diminished	Resistance of a variety of gram-negative bacteria to aminoglycosides
	Altered target	Modification of ribosomal proteins or of 16S rRNA; this reduces the ability of aminoglycoside to successfully bind and inhibit protein synthesis	Resistance of <i>Mycobacterium</i> spp. to streptomycin
Quinolones			
Examples: ciprofloxacin, levofloxacin, norfloxacin, and lomefloxacin	Decreased uptake	Alterations in the outer membrane diminishes uptake of drug or activation of an "efflux" pump that removes quinolones before intracellular concentration is sufficient for inhibiting DNA metabolism	Resistance of gram-negative bacteria and staphylococci (efflux mechanism only) to various quinolones
	Altered target	Changes in DNA gyrase subunits decrease the ability of quinolones to bind this enzyme and interfere with DNA processes	Gram-negative and gram-positive resistance to various quinolones

^a Source: University of Minnesota (41). PBP, penicillin-binding protein.

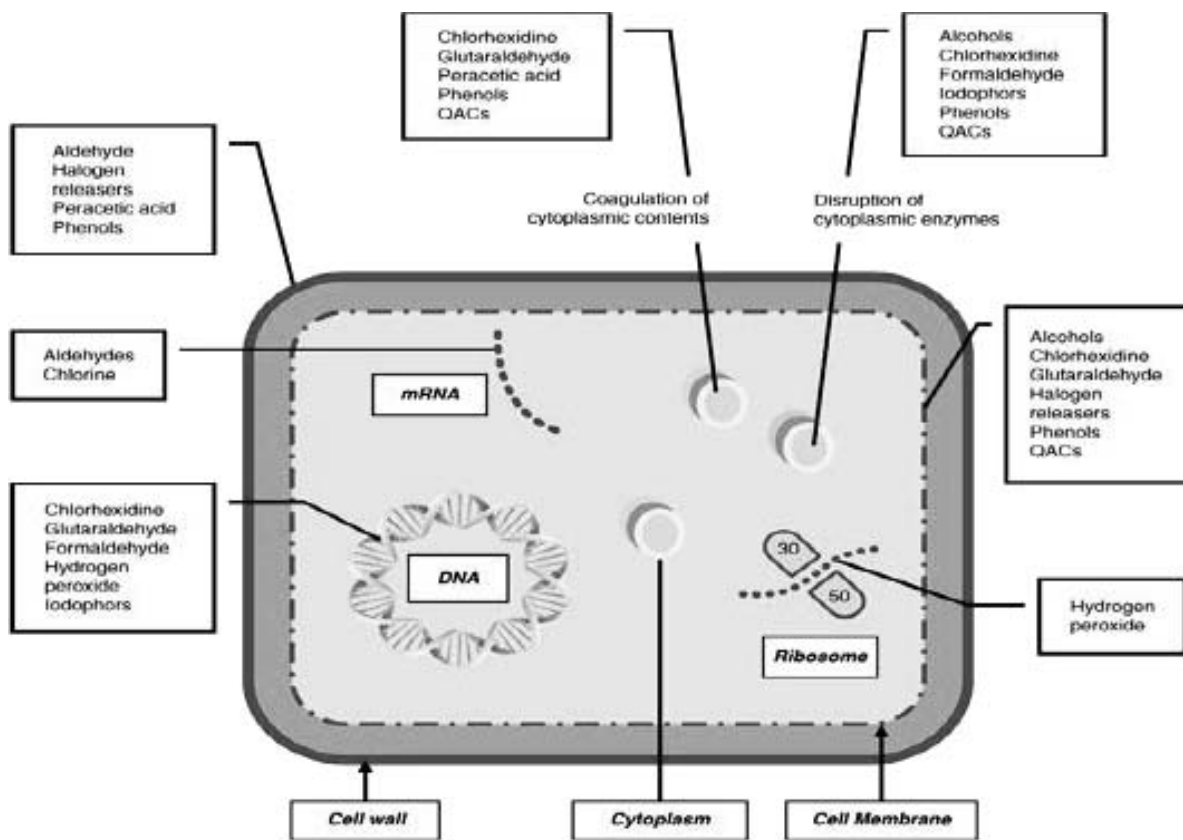


FIGURE 1. Cellular targets of biocidal agents, adapted from Fanning (14).

nonreversible (e.g., through constitutive genetic changes), and bacterial fitness cost associated with resistant phenotypes is high and generally not favored. Although there is experimental evidence to show the possibility of AMR occurrence due to biocide exposure, it is not conclusive enough to demonstrate a causative role. A recent study reported by Kim et al. (24) applied metagenomics and whole genome sequencing techniques to provide strong insights into the mechanistic understanding of biocide exposure resulting in AMR in *Pseudomonas aeruginosa*. The model used in this work may not be readily relevant to food processing facilities; nevertheless, studies such as this may help to shed light on this complex issue.

ASSOCIATION STUDIES DO NOT INFER CAUSALITY

Several reports (4, 29, 39), have used surveillance approaches to recover organisms from food and its associated environment, followed by biocide and AMR characterization of the isolates. Although some have reported the reduced susceptibility to biocides and resistance to antimicrobials of the isolates, these studies do not prove the directionality of the emergence of resistance (i.e., how exposure to biocides coselected for AMR), thus offering weak evidence for causation. To illustrate, Zhang et al. (51) studied *Escherichia coli*-contaminated retail meat samples, concluding that *qac* genes were highly associated with AMR and that they and other disinfectant resistance genes were located on conjugative plasmids. Deng et al. (12) carried out similar research with studied *Salmonella*-

contaminated foods of animal origin, concluding that antibiotic resistance was highly associated with a certain disinfectant or particular heavy metal resistance genes and a proportion of plasmids hosted by *Salmonella* tended to carry the three types of genes. In both cases, the researchers defined disinfectant resistance via MIC and suggested that the presence of genes for multidrug pumps resulted in increased MIC.

Romero et al. (34) used MIC to determine biocide resistance and used the term “biocide tolerance” for strains showing an increased MIC, both of which can be viewed as inappropriate usage of the terminologies. They found a correlation between AMR and increased biocide MIC and concluded there was potential for causality. However, the basis for this is not clear from the data relating to the gene *qacEΔ1*, which is an unspecific multidrug pump.

Peyrat et al. (32) investigated the effects of cleaning and disinfection procedures (including mixtures of QACs and glutaraldehyde or formaldehyde, chlorine-based products) in poultry slaughterhouses, and the potential for these agents to select for AMR in *Campylobacter jejuni* and *Campylobacter coli*. The isolates recovered from equipment surfaces after cleaning and disinfection after five visits to three different slaughterhouses did not show any increase in resistance to antibiotics tested compared with isolates tested before cleaning and disinfection. Thus, association studies provide varied conclusions, and those that demonstrate a plausible link do not offer conclusive causative evidence.

TABLE 2. A summary of selected recent literature (2008 to 2018) investigating the coselection for AMR following exposure to biocides

No.	Microorganism	Overview of study design
1.	<i>Campylobacter jejuni</i> and <i>Campylobacter coli</i> isolated from poultry processing plants as part of this study	Organisms were isolated pre- and postcleaning and disinfection from poultry processing plants; combination of alkali-chlorinated cleaning foam and neutral detergent were used as cleaning agents and quaternary ammonium compound (QAC) plus glutaraldehyde and hexamethylene biguanide were used as disinfectants in the processing plants; predisinfection isolates acted as controls; isolates were evaluated against the following: Disinfectants: benzalkonium chloride and didecyl dimethyl ammonium chloride (DDAC) Antimicrobials: ampicillin, tetracycline, gentamicin, streptomycin, erythromycin, and enrofloxacin
2.	<i>Salmonella</i> Typhimurium	<i>Salmonella</i> Typhimurium exposed for 5 h to low-level and recommended in-use concentrations of four different biocides; following exposure, live cells recovered by using fluorescent-activated cell sorter (FACS) and characterized for antimicrobial resistance; flow cytometer used to study the effect on membrane damage after exposure to biocides Biocides: oxidative compound (Virkon), mixture of aldehydes and QAC (Superkill), QAC (AQAS), and a halogenated tertiary amine compound (Trigene) Antimicrobials: nalidixic acid, chloramphenicol, ciprofloxacin, tetracycline, and kanamycin
3.	A panel of 189 <i>Salmonella</i> strains, including 48 serovars of different origins sourced from a culture collection	Assessment of susceptibility to frequently used food industry biocides and resistance to a panel of antimicrobial agents tested Biocides: seven agents including one alkali, one acid, one QAC, and three mixtures Antimicrobials: 15 clinically important antimicrobials MIC and MBC determined for biocides; break point values determined for antimicrobials
	A subset of six <i>Salmonella</i> strains from the previously mentioned panel chosen for further in vitro selection	Six <i>Salmonella</i> strains exhibiting stable reduced susceptibility to biocides were evaluated for cross-resistance; these six strains showed a higher MIC than the mean MIC for two or more biocide formulation
	<i>Salmonella</i> Enteritidis	Biocide efficacy in presence of biofilm (low and high nutrient) surface dried and planktonic cells evaluated
4.	<i>Salmonella</i> Typhimurium	Type strain repeatedly subcultured for 4 days in presence of sublethal concentration of biocides; aliquots of exposed bacteria serially diluted and plated onto agar plates (original plate), followed by replica plating onto plates containing different levels of antimicrobials; replica of colonies in the original plate that grew in presence of antimicrobials (mutants) were phenotypically and genotypically characterized by whole genome sequencing analysis; fitness cost also investigated Biocides: biocides with different modes of action used. Mixture of aldehyde and QAC, QAC, an oxidative compound, halogenated tertiary amine Antimicrobials: nalidixic acid, chloramphenicol, tetracycline, and kanamycin for mutant selection
5.	<i>Stenotrophomonas maltophilia</i>	Mutants were also selected following exposure to triclosan Molecular characterization of wild-type and mutant strains to understand genetic differences resulting in potential antimicrobial resistance and bacterial fitness assessments to ascertain stability of mutants and evaluation of increased resistance against a panel of antimicrobials

TABLE 2. *Extended*

Condition of organisms (i.e., are they laboratory adapted)	Conclusion	Comment	Reference
Strains isolated from real-life conditions	No selection for AMR observed in isolates obtained postdisinfection	Study in real-life conditions showing no coselection for AMR following the application of cleaning agents and disinfectants	32
Organism exposed to low level and in-use concentrations of biocides	Exposure to low levels of biocide-generated populations with varied health status but selection for AMR not noticed; two mutants survived in-use biocide concentrations recovered by FACS. These mutants exhibited low fitness when compared with wild type (e.g., prolonged lag phase) and were less susceptible to antimicrobials, predominantly conferred through increased efflux pump activity	Application of FACS for recovering mutants is a novelty of this study; exposure to in-use concentrations of biocides, and not low levels, resulted in the selection of mutants exhibiting AMR, in contrast to other reports; FACS replicate experiments with traditional plating methods failed to recover any mutants (i.e., no growth observed at in-use concentrations), potentially indicating that these strains might not survive under natural conditions	48
First part of the study involved no in vitro selection	No correlation between reduced susceptibility to biocides and AMR observed, including 15 individual compounds analyzed	Despite several rounds of in vitro selection, stable reduced susceptibility to biocides under what was likely favorable laboratory conditions for a majority of the isolates was not achieved, potentially indicating the rarity of this phenomenon in real-life conditions	9
Repeated exposure to biocides resulted in the development of heritable phenotypes showing reduced susceptibility	No shared insusceptibility observed against biocides; changes in susceptibility to antimicrobial agents noticed (three strains showed reduced susceptibility and one was increased)	Shared insusceptibility to biocides is mostly unlikely, given the high concentrations used and the multiple mechanisms of action of these agents against bacterial cells; cross-resistance to antimicrobials is a possibility, although the chances of likelihood are very limited; the mechanism behind “heritable/stable” phenotype is not understood	
This strain was selected as it exhibited an increased capacity to form biofilms under laboratory conditions	Planktonic organisms were the most susceptible; reduced susceptibility to biocides noticed when cells were in biofilm or dried surface	Cleaning and disinfection steps in food processing facilities must follow recommended protocol to minimize sustained exposure to sublethal concentration of sanitizers	
Organism exposed to sublethal concentrations of four different biocides to select for mutants.	Biocide exposure resulted in selection of stable mutants exhibiting increased MICs to antimicrobial agents, and no resistance to biocides observed; exposure to biocide induced mutations only in seven genes conferring resistance; fitness of mutants and wild-type strains were comparable	Study provides mechanistic details on coresistance for AMR following exposure to sublethal concentrations of biocides in laboratory-adapted strain; fate of control cells not clearly discussed	47
A laboratory strain is exposed to sublethal concentrations of benzalkonium chloride and hexachlorophene to select mutants	AMR not associated with exposure to hexachlorophene, resistance against some antimicrobials noticed with benzalkonium chloride (BAC); bacterial fitness reduces on acquisition of resistance, and these populations are potentially not stable	Although these results provide some evidence for AMR through cross-resistance mechanisms (increased efflux pump activity), results from bacterial fitness assessments show that these phenotypes are not favored; therefore, it is reasonable to conclude that cross-resistance to antimicrobials in real-life conditions at recommended in-use biocidal concentrations will be negligible	36

TABLE 2. *Continued*

No.	Microorganism	Overview of study design
6.	<i>Escherichia coli</i> ($n = 54$), <i>C. coli</i> ($n = 16$), <i>Salmonella</i> ($n = 35$), and <i>Listeria monocytogenes</i> ($n = 31$) isolated from pig feces	Laboratory-adapted isolates tested for increased MICs to antimicrobials and biocides Biocide: DDAC Antimicrobials: a panel of antimicrobials used for each species, 20 for <i>L. monocytogenes</i> , 14 for <i>Salmonella</i> , 7 for <i>C. coli</i> , and 13 for <i>E. coli</i>
7.	<i>Salmonella</i> Typhimurium	Genomic and transcriptomic analysis of mutants and fitness cost evaluated; overall results correlated with 16 different field isolates that intrinsically showed reduced susceptibility to biocides Biocides: triclosan, BAC, chlorhexidine, and sodium hypochlorite Antimicrobials: ampicillin and ciprofloxacin
8.	<i>C. jejuni</i>	Biocide-adapted strains were assessed for (i) their biofilm-forming capabilities and (ii) their reduced susceptibility to antimicrobial agents in comparison to wild type Biocides: trisodium phosphate, acetic acid, sodium hypochlorite, and two commercial biocide preparations Antimicrobials: ampicillin, chloramphenicol, tetracycline, erythromycin, ciprofloxacin, nalidixic acid, kanamycin, streptomycin, and gentamicin
9.	A panel of 76 strains isolated from organic foods and belonging to the genera <i>Bacillus</i> sp., <i>Enterococcus</i> sp., <i>Staphylococcus</i> sp., <i>Enterobacter</i> sp., <i>Chryseobacterium</i> sp., <i>Klebsiella</i> sp., <i>Pantoea</i> sp., and <i>Salmonella</i>	Pairwise mutant and wild-type strain evaluated for susceptibility to biocides and antimicrobials; growth characteristics and heat tolerance were also analyzed along with PCR-based detection of resistance genes Biocides: benzalkonium chloride, hexadecylpyridinium chloride, cetrimide, triclosan, chlorhexidine, and hexachlorophene Antimicrobials: ampicillin, cefotaxime, ciprofloxacin, imipenem, ceftazidime, tetracycline, trimethoprim/sulfamethoxazole, and nalidixic acid
10.	<i>Pseudomonas aeruginosa</i>	Microbial community maintained in aerobically fed batch bioreactors; metagenomics on entire microbial communities and whole genome sequencing of <i>P. aeruginosa</i> isolate performed to deduce causality between BAC exposure and promotion of AMR

USE OF LABORATORY-ADAPTED STRAINS IN RESEARCH STUDIES THAT INFER CAUSALITY

A different approach to association studies has been taken by some researchers to investigate the causative role of biocide exposure resulting in antimicrobial resistance. The majority of these studies have used laboratory strains exposed to sublethal concentrations of biocides. Unsurpris-

ingly, this preconditioning reduces the susceptibility of these strains, often resulting in reduced susceptibility to biocides and increased MICs for antimicrobial agents (Table 2).

Condell et al. (9) investigated the tolerance of a collection of susceptible and multidrug-resistant *Salmonella enterica* to seven commercially available food-grade biocide formulations in vitro, exploring their abilities to

TABLE 2. Continued, Extended

Condition of organisms (i.e., are they laboratory adapted)	Conclusion	Comment	Reference
Isolates exposed to increasing sublethal levels of DDAC, a QAC, for 7 days	MICs to biocides were stable or increased, depending on organism/biocide combination following adaptation; coselection for antimicrobial (increased MIC) resistance pronounced in <i>E. coli</i>	Study demonstrates increased MIC in limited organisms to certain antimicrobials by using laboratory conditions that may not be applicable to real-life situations	38
<i>Salmonella</i> Typhimurium exposed to subinhibitory concentrations of biocides and antimicrobial agents to select stable mutants	Laboratory-derived mutants showed variable susceptibility to antimicrobials; mutants exhibited both genetic and phenotypic adaptations and were less fit in comparison to the wild type	Biocide application at recommended concentrations will be lethal to the organisms and, in general, will not favor coselection in real-life conditions	11
A laboratory-type strain adapted to increasing concentrations of biocides	Laboratory-adapted strains showed minimal increase in MICs, to a few biocides, and antimicrobial agents; although some adapted strains showed improved biofilm-forming capacity, adaptation to trisodium phosphate reduced biofilm formation	Increased MICs to biocides is inconsequential at recommended in-use concentration; although coselection of resistance is reported for a few antimicrobials, it has been demonstrated by using laboratory-adapted strains	40
Strains were originally biocide sensitive. Susceptibility reduced by exposure to QACs to select stable phenotypes	In vitro-adapted strains can exhibit varied susceptibility to different antimicrobials and biocides; reduced susceptibility can be due to physiological adaptations or genetic changes	Changes in susceptibility to biocides and antimicrobials on different laboratory-adapted organisms is shown though its significance in real-life conditions is limited	18
A river sediment sample maintained for 3 yr in aerobically fed batch bioreactors, with and without exposure to BAC, with an additional carbon source and only BAC; <i>P. aeruginosa</i> isolated from these reactors was also adapted under laboratory conditions by exposure to increasing concentrations of BAC to even more than recommended in-use levels	Study demonstrates multiple mechanisms, including constitutive genetic changes, through which exposure to BAC can result in resistance to antimicrobials, such as polymyxin, tetracycline, and ciprofloxacin, in <i>P. aeruginosa</i>	This study provides causative evidence for reduced susceptibility of <i>P. aeruginosa</i> to certain antimicrobials through long-term exposure to sublethal levels of BAC; however, contrasting results were obtained with other antimicrobials; conditions simulated in the bioreactor may not exist in real-life food processing settings (i.e., prolonged long-term exposure to sustained sublethal levels of BAC that can result in “exposed” strains that were later also used in adaptation experiments)	24

adapt to those formulations and their active biocidal agents, including BAC, after sequential rounds of in vitro selection. A stable phenotype of tolerance to the studied formulations could not be selected. Upon exposure of *Salmonella* to an active biocidal compound, a high-level of tolerance was selected for a number of serotypes. No coselection to the different biocidal agents or food-grade formulations was observed. Most tolerant isolates displayed variable changes

in their patterns of susceptibility (increase or decrease) to antimicrobial compounds. In contrast to these findings, another study reported no changes in the AMR response of *Salmonella* Typhimurium following exposure to sublethal concentrations of four different biocides (48). Although the authors reported the recovery of a few mutants exhibiting increased MICs to some antimicrobials following exposure to in-use biocide concentrations by a fluorescent-activated

cell-sorting technique, the results were not replicated with traditional methods, potentially indicating the low survivability of such mutants under natural conditions.

Sanchez et al. (36) exposed *Stenotrophomonas maltophilia*, a water treatment-relevant organism to hexachlorophene and BAC to generate mutants. Molecular and phenotypic characterization of these mutants showed resistance against some antimicrobials on exposure to BAC, and not hexachlorophene, through increased efflux pump activities (cross-resistance). A different study reported pronounced coselection for AMR in *E. coli* following exposure to increasing sublethal doses of dodecyl dimethyl ammonium chloride, a QAC, when compared with *C. coli*, *Salmonella*, and *L. monocytogenes* isolated from pig feces (38). Although these and a few other reports (11, 19, 40) demonstrate the in vitro likelihood of occurrence of biocide-induced coselection for AMR, the consequences of these findings are of limited real-life significance in food-manufacturing conditions. This is mainly for the following:

1. biocides are used at much higher concentrations in food hygiene applications than MIC levels, and
2. although laboratory-adapted strains provide a practical approach for investigating the coselection phenomenon, these experiments using “trained strains” (46) at best simulate the consequences of low traces of the environmental presence of nondegradable biocides.

In general, we believe that this situation is less likely to be encountered under real-life conditions, especially when validated cleaning and disinfection procedures are followed.

ALTERED RESPONSE TO AN ANTIMICROBIAL CAN BE TRANSIENT OR STABLE

The altered response to antimicrobial agents due to biocide exposure can be either transient (e.g., through phenotypic adaptation) or stable (e.g., through heritable genetic changes). Bacteria can exhibit intrinsic or acquired resistance to both biocides and antimicrobial agents. For instance, gram-negative organisms and mycobacteria are intrinsically less sensitive to the effects of biocides due to the nature of their outer cell wall membrane and cell wall, respectively. Acquired resistance can be caused by mutations, acquisition of mobile genetic elements, such as plasmids, or through altered gene expression (30). Increased efflux pump activity (48), a common feature in both reduced biocide susceptibility and antimicrobial resistance, can be either intrinsic or acquired through plasmid transfer or genetic mutations. In-depth discussions of these mechanisms is beyond the scope of this article, and readers are referred to Wales and Davies (46) for an excellent review on this topic. Note that resistance conferred through altered gene expression is transient and often wanes with the removal of the stressor (biocide) posing minimal risk for the coselection of stable antimicrobial-resistant populations (21). On the contrary, genetic mutations resulting in constitutively expressing mutants (24, 47) and acquisition of mobile genetic elements (24), following exposure to biocides, can result in increased antimicrobial resistance. Kim et al. (24) have recently shown the stable acquisition of genetic islands and mutations in *prmB* gene of *P.*

aeruginosa resulting in AMR following exposure to BAC in bioreactors or through laboratory adaptation experiments by exposing them to levels even higher than the recommended in-use concentrations.

Studies have also shown that a cost is incurred toward the acquisition of antimicrobial-resistant phenotypes following exposure to sublethal levels of biocides in most of the cases (Table 2), and such phenotypes may not be readily favored over the wild types (11, 36, 48), while no change in fitness has been reported (47). Thus, the majority of these reports that have investigated acquired resistance mechanisms have used laboratory-adapted strains potentially limiting the relevance of their findings to real-life conditions (26). In addition, in most of the earlier studies that have used laboratory-adapted strains to report the altered MIC response to antimicrobials following biocide exposure (9, 19, 36), it is difficult to discern the mechanisms behind the generation of stable populations exhibiting such differences (i.e., differentiation between phenotypic adaptation and constitutive genetic change is generally lacking). Although improved access to whole genome sequencing techniques and bioinformatics tools enables some of the recent studies (24, 47) to address this critical difference, further widespread research in this area by using genomics approaches is warranted.

META-ANALYSIS STUDIES ON EVIDENCE OF CAUSALITY BETWEEN BIOCIDES EXPOSURE AND SELECTION FOR AMR

A number of meta-analysis studies and expert reviews on biocide-induced coselection for AMR in bacteria have been published, and a select comprehensive few are summarized in Table 3. In general, most of the reviews conclude on the lack of a strong causal evidence to link biocide exposure with the emergence or spread of AMR. On the basis of the existing theoretical evidence, a few of these reports propose recommendations on the basis of the “precautionary principle” to prevent such occurrences. While supporting most of these recommendations, we also encourage research that is relevant to real-life conditions to understand and quantify the effect of biocide-induced coselection for AMR.

BIOFILMS: A LESSER STUDIED CONTRIBUTOR

Biofilms can occur in food processing environments and are known to offer protection to microorganisms against cleaning and disinfectant agents by reducing or preventing their access through the presence of exopolysaccharides (10). The nutrient-deprived state of the organisms within the biofilms acts as additional stressors, and their proximity to each other can favor genetic exchanges (19). However, the majority of the studies investigating biocide-induced coselection for AMR have focused on using planktonic cells (Table 2), and those findings may not be applicable to biofilms, though it is likely that organisms within biofilms can withstand a robust chemical challenge (9), thus favoring coselection. The mechanisms behind the formation of biofilms is poorly understood, and it appears that the type of biocide used itself can promote or even minimize the

TABLE 3. A summary of select meta-analysis and review studies on biocide-induced coselection of antimicrobial resistance

No.	Conclusion or relevant comment	Reference	Comment or recommendation
1.	The European Scientific Committee on Emerging and Newly Identified Health Risks noted that common resistance mechanisms to biocides and antimicrobials could occur; in addition, widespread regular usage of biocides can maintain continuous selective pressure and thus increase the risk for coselection	37	This issue is dealt with in practice by the development and provision of usage instructions to assure exposure to appropriate (biocidal) concentrations and contact times; recommendations made in the opinion for further work have since been acted on by the European Commission (e.g., provision of data on microbial resistance as part of the biocide approval process) and researchers
2.	The European Union BIOHYPO project studied relationship between reduced susceptibility to biocides and AMR in a large data set of <i>Salmonella</i> and <i>Staphylococcus aureus</i> ; this project concluded that no evidence exists to link biocide usage to increased antimicrobial resistance	4	The conclusion of this project supports the findings of our analysis of the evidence available up-to-date
3.	Authors note the existence of contradictory data on biocide exposure helping to maintain antimicrobial-resistant bacteria and suggest the use of biocidal solutions containing more than one active ingredient, at recommended concentrations and rotation of biocidal products; biofilms are identified as a threat for disinfection, and the authors encourage specific monitoring programs to determine efficacy of disinfection and monitor possible persistence and spread of biocide and antimicrobial-resistant bacteria	30	There is a paucity of scientific evidence to (i) stop the use of biocides containing a single active ingredient and (ii) rotate disinfectant products to limit the emergence/spread of coselection
4.	This review concludes on the nonexistence of a consistent pattern between antimicrobial and biocide resistance; in addition, they note that transfer of resistance mechanisms resulting from biocide exposure is not yet demonstrated	45	The major conclusions of this review are in alignment with our assessment of the available scientific evidence
5.	In an extensive meta-analysis study, the authors acknowledge the existence of limited scientific evidence for coselection; however, they highlight the use of laboratory-adapted strains in most of the studies for gathering such evidence and how it does not correlate with real-world situation; they also contend that the long use of biocides in several settings, such as health care, agriculture, and industry, has not resulted in a generalized problem	46	The findings of this in-depth meta-analysis correlates well with our conclusions
6.	A recent Joint Food and Agriculture Organization/World Health Organization Expert Committee in collaboration with World Organization for Animal Health noted that biocides have the potential to coselect for AMR, evidence gained mostly through in vitro studies, while also highlighting the major research gaps that needs to be addressed; notably, studies need to consider the role of surfactants, sequestrants, and other compounds that are normally present along with biocides in their evaluation, quantify the AMR attributable to biocide exposure and develop standardized methodologies for measuring/monitoring resistance; the committee recommends proactive measures to be undertaken despite the current knowledge gaps to minimize coselection	17	In addition to adhering to the recommended biocide usage practices as has been suggested by the committee, we are also in favor of closing the knowledge gaps identified and encourage research to identify measures to (i) inactivate residual biocides before introducing in wastewater and (ii) assess potential of new biocides for coselection against clinically important antimicrobials

biofilm-forming potential of an organism (40), highlighting the knowledge gaps to be addressed to understand the role in biocide-induced coselection of AMR.

Araújo et al. (3) reported the problems of biofilm formation and on the main strategies used for control,

together with information on the antimicrobial mode of action of biocides and biofilm resistance mechanisms. They noted that the chemical control of biofilms is an important issue because of its complexity and because resistance to biocides is unavoidable.

Bridier et al. (5) reviewed mechanisms identified as playing a role in biofilm resistance to disinfectants, as well as novel antibiofilm strategies. They commented that the definition of “resistance” needs to be clarified, as it changes depending on whether planktonic or biofilm cells are considered. From the studies reviewed in this article, the authors mainly attributed biofilm resistance to disinfectants to the three-dimensional structure and heterogeneous nature of the biofilms along with other multifactorial mechanisms. The authors also call for regulatory standards focusing on assessing the efficacy of a disinfectant to take into account the “mode of life” of biofilms (i.e., efficacy testing must include nonplanktonic cells, a position that we support).

CONCLUSIONS AND RECOMMENDATIONS

It is important that consistent terminology be used between studies to describe the alteration in bacterial phenotype in response to exposure of antibiotics or other antimicrobial agents or both.

Much early work had ambiguity regarding phenomena and terminology. For example, the term “resistance” has often been used to describe erroneously an increase in MIC or an increase in MBC, following exposure to biocides. This has resulted in a lack of clarity between phenotypic adaptation and stable resistance, and conclusions drawn with respect to antibiotic resistance that are not borne out by some of the data.

A long history of biocide use in health care, agriculture, and food industry has not shown any consistent evidence of AMR to such agents. Conversely, sufficient evidence exists of a causal relationship between the risk of antibiotic resistance and the relatively recent introduction of antibiotics in clinical treatment and for livestock production and disease treatment.

The mode of biocide usage and design to deliver a microbial kill, compared with antibiotics, would appear to mitigate against survival of a subpopulation of bacteria and subsequent development of a lowered susceptibility. Biocides predominantly act on multiple bacterial structures or metabolic functions; hence, there is a limited risk for the development of multiple-resistant processes that would render antibiotics ineffective, given their targeted mode of action.

Disinfectants and sanitizers are often complex formulations, containing one or more biocides and a number of excipients or adjuncts that potentiate the activity of the biocides or have some activity of their own. Note that concentrations of chemicals at MICs, which can allow phenotypic adaptation to occur, are significantly below those levels recommended to be used in practice in food hygiene (i.e., using disinfectants at concentrations and contact times determined by accepted efficacy testing). However, inappropriate application (resulting in possible dilution of active agents) of biocide use or ineffective access to microbial populations (e.g., through heavy organic soiling on surfaces) may present a risk scenario for AMR (9, 20, 30, 35). In addition, there may be potential increased risk of AMR coselection with biocides having a narrow or single mode of action. The proper application of biocides or sanitizing agents, according to manufacturers’ instructions,

is advised, and cleaning programs should consider worst-case scenarios as part of cleaning validations. Verification and monitoring activities should include visual inspection or other means to ensure removal of proteinaceous materials or organic soiling during cleaning and that the effective concentration or contact time of the biocide has been applied.

Theoretically, coselection between antibiotics and biocidal agents is a possibility. Laboratory studies conducted with various planktonically grown foodborne pathogens, including *Salmonella*, *L. monocytogenes*, and *Campylobacter* spp., have used various biocides at sublethal or increasing concentrations to demonstrate cross- or coresistance to various antibiotic groups. However, the in vitro use of concentrations not typical of in-use levels and the phenomenon of training of field or laboratory strains has been speculated as being unrepresentative of field situations (46).

In situ research that has been reported (e.g., in hospital settings) has not conclusively demonstrated sustained antibiotic resistance despite severalfold changes in MIC when biocide chemicals are used appropriately. However, phenotypic adaptation and stable resistance in some instances has been demonstrated after exposure to biocidal agents at sublethal concentrations.

This review of key individual studies and meta-analysis studies lacks substantive evidence that the appropriate use of chemicals for food hygiene promotes antimicrobial resistance. Limited data of food production scenarios—primary production, primary processing, manufacturing, retail, catering, and consumer home—on the ecology of foodborne microorganisms (pathogens, spoilage microorganisms, and general microflora), before and after biocide application, exist to demonstrate first, their persistence or survival and second, whether such microorganisms are at risk of acquiring reduced antibiotic susceptibility. Further research is recommended to explore the potential for resistance to occur in “real or in situ” food production environments in which the appropriate usage is being made of chemicals for food hygiene or where challenging cleaning or disinfection scenarios arise. Phenotypic adaptation can be minimized in practice by applying good manufacturing practices to rigorous cleaning and disinfection, using concentrations of sanitizers and disinfectants and contact times, as recommended by the manufacturer, as appropriate to control target microorganisms.

Although cleaning regimes tend to have a final water rinse step (unless for rinse-free formulations), this can lead to traces of biocidal agents in wastewater. In food production facilities, efforts should be made to avoid biocide residues entering the wastewater streams, where practically possible. However, a mitigation aspect is that often the wastewater contains organic matter, which can render the sublethal amounts of biocide agent ineffective (45).

Biofilms present a unique challenge for efficacy of biocides, even at recommended in-use concentrations. Therefore, food manufacturer sanitation programs should consider the likelihood of biofilm formation and adjust cleaning regimes accordingly, while ensuring hygienic

design of equipment to minimize biofilm formation together with the use of validated cleaning and disinfection procedures and application of appropriate monitoring and verification measures. Manufacturers of biocide or sanitizing agents should also consider improvements in active agents or supplementary adjuncts to ensure biofilms first, must not become a hygienic food safety issue and second, that there are no unintended consequences with respect to survivors acquiring reduced susceptibility to antibiotics.

Manufacturers of biocidal agents should consider assessing the potential (e.g., through *in silico* studies) of new biocide products for potential to lead to coselection to clinically relevant antibiotics. The authorization of new biocidal active substances must take a scientific and risk-based approach regarding the potential for driving microbial resistance.

Biocides or sanitizing agents and heavy metals are often grouped together as agents leading to coselection of antibiotic resistance. However, note that heavy metals, when used in agricultural production, are used at sublethal supplementary levels. This is distinct from biocides that are in the main applied at killing or inactivation concentrations. Therefore, a clear distinction should be made between potential risk of acquired antibiotic cross-resistance between metal ions and antibiotics and the risk posed by biocides.

Furthermore, beyond the potential impact of biocide usage, limitation of the use of antimicrobials in primary agricultural production of plant and animal food products is the global priority action to minimize potential for AMR resistance and should be achieved by adherence to good agricultural practice and good veterinary drug practice.

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