LITTERATURE REVIEW ON THE DEVELOPMENT OF (CROSS-)RESISTANCES TO ANTIMICROBIALS FOLLOWING THE USE OF BIOCIDAL PRODUCTS

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The emergence of resistance after the use of biocidal products

Summary of the report

Resistance to antimicrobials is a growing worldwide issue that may spiral out of control if no action is taken to prevent its spread. An effective solution to control microorganisms is to prevent colonization on surfaces by using disinfectants and other biocidal products. But while focus has mainly been on the development of resistance following the use of antibiotics, much less is known about how microorganisms develop resistance following the use of biocidal products. Biocidal products are those that are intended to destroy, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means. Microorganisms are usually considered resistant when they survive exposure to a product which would normally kill them or stop their growth. One major concern is that microorganisms which survive exposure to a biocidal product develop resistance mechanisms that also provide protection against antibiotics used to treat human infection.

This report aims to review the literature pertaining to the resistance and cross-resistance of microorganisms to biocidal products belonging mostly to categories PT1 and PT2. Due to the plethora of biocidal products that are available, this report focuses on the risk associated with the development of resistance and cross-resistance following the use of the following active substances: alcohols, aldehyde-based compounds, hydrogen peroxide, peracetic acid, chlorhexidine, quaternary ammonium compounds, chlorine releasing compounds and weak organic acids. Although not authorized in the EU, triclosan was also included as it constitutes an interesting case study. This report focuses on bacteria, as they are the subject of the vast majority of studies on resistance to biocides. Available data on mycobacteria, yeasts and molds were included when available. Since viruses only replicate (and mutate) within the host, resistance following disinfection with a biocidal product is highly unlikely to happen. Accordingly, no data on this subject was found in the literature.

After analyzing the relevant literature, we found that there is a large amount of data that supports a role for biocidal products in the emergence of resistance to antimicrobials, but the importance of this role largely depends on the type of biocidal product used, the microorganism affected and the method and setting in which the biocidal product was used.

Concerning the risk of development of resistance following the use of biocidal substances, we found that alcohols, hydrogen peroxide, peracetic acid and weak organic acids constitute a highly unlikely risk, aldehyde-based products and chlorine releasing agents constitute an unlikely risk, chlorhexidine and quaternary ammonium compounds constitute a likely risk and triclosan constitutes a highly likely risk. The microorganisms affected are diverse: Gram-positive and Gram-negative bacteria, mycobacteria and yeasts, although most of the data available relates to bacteria. The use of chlorhexidine, quaternary ammonium compounds and triclosan mainly is strongly associated with the development of cross-resistance to other antimicrobials, including antibiotics such as tetracycline, vancomycin, chloramphenicol, ciprofloxacin, imipenem and colistin.

This report also references many gaps of knowledge on the subject, including the lack of standardized biocide testing protocols and comprehensive studies for understanding resistance to biocidal products in practice, not just in the lab. We also recommend implementing a surveillance program and to restrict, whenever possible, the use of biocidal products such as chlorhexidine, quaternary ammonium compounds and triclosan that are associated with the development of resistance and cross resistance.

Résumé du rapport

La résistance aux antimicrobiens est un problème mondial croissant qui pourrait devenir incontrôlable si aucune mesure n'est prise pour empêcher sa propagation. Une solution efficace pour lutter contre les micro-organismes consiste à empêcher la colonisation des surfaces en utilisant des désinfectants et d'autres produits biocides. Mais si l'on s'est surtout intéressé au développement de la résistance après l'utilisation d'antibiotiques, on sait beaucoup moins comment les micro-organismes développent une résistance après l'utilisation de produits biocides. Les produits biocides sont ceux qui sont destinés à détruire, à rendre inoffensif, à empêcher l'action ou à exercer un effet de contrôle sur tout organisme nuisible par des moyens chimiques ou biologiques. Les micro-organismes sont généralement considérés comme résistants lorsqu'ils survivent à l'exposition à un produit qui devrait normalement les tuer ou arrêter leur croissance. Une préoccupation majeure est que les micro-organismes qui survivent à l'exposition à un produit biocide développent des mécanismes de résistance qui assurent également une protection contre les antibiotiques utilisés pour traiter les infections humaines.

Ce rapport a pour but d'examiner la littérature relative à la résistance et à la résistance croisée des micro-organismes aux produits biocides appartenant principalement aux catégories PT1 et PT2. En raison de la pléthore de produits biocides disponibles, ce rapport se concentre sur le risque associé au développement de la résistance et de la résistance croisée suite à l'utilisation des substances actives suivantes : les alcools, les composés à base d'aldéhydes, le peroxyde d'hydrogène, l'acide peracétique, la chlorhexidine, les composés d'ammonium quaternaire, les composés libérant du chlore et acides organiques faibles. Bien que non autorisé dans l'UE, le triclosan a également été inclus car il constitue une étude de cas intéressante. Ce rapport se concentre sur les bactéries, car elles font l'objet de la grande majorité des études sur la résistance aux biocides. Les données disponibles sur les mycobactéries, les levures et les moisissures ont été incluses lorsqu'elles étaient disponibles. Étant donné que les virus ne se répliquent (et ne mutent) qu'à l'intérieur de l'hôte, il est très peu probable qu'une résistance se produise après une désinfection avec un produit biocide. Par conséquent, aucune donnée sur ce sujet n'a été trouvée dans la littérature.

Après avoir analysé la littérature pertinente, nous avons constaté qu'il existe un grand nombre de données qui soutiennent un rôle des produits biocides dans l'émergence de la résistance aux antimicrobiens, mais l'importance de ce rôle dépend largement du type de produit biocide utilisé, du micro-organisme affecté et de la méthode et du contexte dans lesquels le produit biocide a été utilisé.

Nous avons constaté qu'en ce qui concerne le risque de développement d'une résistance suite à l'utilisation de substances biocides, les alcools, le peroxyde d'hydrogène, l'acide peracétique et les acides organiques faibles constituent un risque très improbable, les produits à base d'aldéhyde et les agents de libération de chlore constituent un risque improbable, la chlorhexidine et les composés d'ammonium quaternaire constituent un risque probable et le triclosan constitue un risque très probable. Les micro-organismes concernés sont divers : bactéries à Gram positif et à Gram négatif, mycobactéries et levures, bien que la plupart des données disponibles concernent les bactéries. L'utilisation de la chlorhexidine, des composés d'ammonium quaternaire et du triclosan est principalement associée au développement d'une résistance croisée à d'autres antimicrobiens, notamment des antibiotiques tels que la tétracycline, la vancomycine, le chloramphénicol, la ciprofloxacine, l'imipénème et la colistine.

Ce rapport fait également état des nombreuses lacunes dans les connaissances sur le sujet, notamment l'absence de protocoles d'essai normalisés des biocides et d'études complètes permettant de comprendre la résistance aux produits biocides dans la pratique, et pas seulement en laboratoire. Nous recommandons également de mettre en place un programme de surveillance et de limiter, dans la mesure du possible, l'utilisation de produits biocides tels que la chlorhexidine, les composés d'ammonium quaternaire et le triclosan, qui sont associés au développement de la résistance et de la résistance croisée.

Samenvatting van het verslag

Resistentie tegen antimicrobiële stoffen is een groeiend wereldwijd probleem dat uit de hand kan lopen als er geen actie wordt ondernomen om de verspreiding ervan te voorkomen. Een doeltreffende oplossing om micro-organismen onder controle te houden is het voorkomen van kolonisatie op oppervlakken door het gebruik van ontsmettingsmiddelen en andere biociden. Maar terwijl de aandacht vooral is gericht op de ontwikkeling van resistentie als gevolg van het gebruik van antibiotica, is veel minder bekend over hoe micro-organismen resistentie ontwikkelen door het gebruik van biociden. Biociden zijn producten die bedoeld zijn om een schadelijk organisme langs chemische of biologische weg te vernietigen, onschadelijk te maken, de effecten ervan te voorkomen of op andere wijze te beheersen. Micro-organismen worden gewoonlijk als resistent beschouwd wanneer zij de blootstelling overleven aan een product dat hen normaal zou doden of hun groei zou stoppen. Een belangrijk punt van zorg is dat micro-organismen die blootstelling aan een biocide overleven, resistentiemechanismen ontwikkelen die ook bescherming bieden tegen antibiotica die worden gebruikt om infecties bij de mens te behandelen.

In dit verslag wordt een overzicht gegeven van de literatuur met betrekking tot de resistentie en kruisresistentie van micro-organismen tegen biociden die voornamelijk tot de categorieën PT1 en PT2 behoren. Gezien de overvloed aan biociden die beschikbaar zijn, wordt in dit verslag vooral ingegaan op het risico van de ontwikkeling van resistentie en kruisresistentie na het gebruik van de volgende werkzame stoffen: Alcoholen, op aldehyden gebaseerde verbindingen, waterstofperoxide, perazijnzuur, chloorhexidine, quaternaire ammoniumverbindingen, chloorafgevende verbindingen en zwakke organische zuren. Hoewel het in de EU niet is toegestaan, werd ook triclosan opgenomen, omdat het een interessante casestudie vormt. Dit verslag is toegespitst op bacteriën, aangezien het merendeel van de studies over resistentie tegen biociden op bacteriën betrekking heeft. Ook de beschikbare gegevens over mycobacteriën, gisten en schimmels zijn opgenomen. Aangezien virussen zich alleen binnen de gastheer vermeerderen (en muteren), is het hoogst onwaarschijnlijk dat resistentie optreedt na desinfectie met een biocide. In de literatuur werden hierover dan ook geen gegevens gevonden.

Na analyse van de relevante literatuur hebben wij vastgesteld dat er een grote hoeveelheid gegevens is die een rol van biociden bij het ontstaan van resistentie tegen antimicrobiële stoffen ondersteunt, maar dat de omvang van deze rol grotendeels afhangt van het gebruikte type biocide, het getroffen micro-organisme en de methode en de setting waarin de biocide is gebruikt.

Wat betreft het risico van resistentieontwikkeling na het gebruik van biociden, hebben wij vastgesteld dat alcoholen, waterstofperoxide, perazijnzuur en zwakke organische zuren een zeer onwaarschijnlijk risico vormen, dat op aldehyde gebaseerde producten en chloorafgevende agentia een onwaarschijnlijk risico vormen, dat chloorhexidine en quaternaire ammoniumverbindingen een waarschijnlijk risico vormen en dat triclosan een zeer waarschijnlijk risico vormt. De getroffen micro-organismen zijn divers: Gram-positieve en Gram-negatieve bacteriën, mycobacteriën en gisten, hoewel de meeste beschikbare gegevens betrekking hebben op bacteriën. Het gebruik van chloorhexidine, quaternaire ammoniumverbindingen en triclosan wordt vooral in verband gebracht met de ontwikkeling van kruisresistentie tegen andere antimicrobiële stoffen, waaronder antibiotica als tetracycline, vancomycine, chlooramfenicol, ciprofloxacine, imipenem en colistine.

In dit verslag wordt ook gewezen op vele lacunes in de kennis over dit onderwerp, waaronder het ontbreken van gestandaardiseerde testprotocollen voor biociden en uitgebreide studies om inzicht te krijgen in de resistentie tegen biociden in de praktijk, niet alleen in het laboratorium. Ook wordt aanbevolen een bewakingsprogramma uit te voeren en waar mogelijk het gebruik te beperken van biociden zoals chloorhexidine, quaternaire ammoniumverbindingen en triclosan, die in verband worden gebracht met de ontwikkeling van resistentie en kruisresistentie.

List of abbreviations

Abbreviation	Definition
ABC	ATP-Binding Cassette
ACP	Acyl Carrier Protein
AMR	Antimicrobial resistance
ATP	Adenosine triphosphate
ATR	Acid Tolerant Response
CEP	Panel on Food Contact Materials, Enzymes and Processing Aids
CFU	Colony Forming Units
EFSA	European Food Safety Authority
ENR	Enoyl-acyl Carrier Protein Reductase
EPS	Exopolysaccharides
EU	European Union
FHL	Formate Hydrogen Lyase
GHP	Good Hygiene Practices
GNB	Gram-negative bacilli
HBV	Hepatitis B virus
HIV	Human Immunodeficiency Virus
LPS	Lipopolysaccharide
MATE	Multidrug and Toxin Extrusion
MBC	Minimum Bactericidal Concentration
MDK	Minimum Duration for Killing
MDR	Multidrug Resistant
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MRSE	Methicillin-Resistant *Staphylococcus epidermidis*
OMP	Outer-membrane Protein
PACE	Proteobacterial Antimicrobial Compound Efflux
RND	Resistance-Nodulation-cell Division
ROS	Reactive Oxygen Species
SARS	Severe Acute Respiratory Syndrome
SMR	Small Multidrug Resistance
SOD	Superoxide Dismutase
TB	Tuberculosis
VBNC	Viable but non-culturable
WHO	World Health Organization
WT	Wild-type

General introduction

Emergence of resistance to antimicrobials and infections caused by bacteria and AMR bacteria

Resistance to antimicrobials is a growing worldwide issue. It was estimated that, in total, about 700,000 people die every year from drug-resistant strains of common bacterial infections, HIV, TB and malaria (1). This number is expected to rise to more than 10 million by 2050 if no action is taken to prevent the spread of antimicrobial resistance (1). An effective solution to fight microorganisms is to prevent colonization on surfaces by using disinfectants and other biocidal products. But while a major amount of attention has been devoted to the development of resistance following the use of **antibiotics** (chemotherapeutic drugs designed to eradicate an infection in humans or animals), much less is known about how microorganisms develop resistance following the use of **biocidal products**.

According to the Biocides Regulation (EU) No 528/2012, biocidal products are those that are intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by any means other than mere physical or mechanical action. Examples include **disinfectants, preservatives**, **antiseptics**, **fungicides** and **insecticides** (2). Microorganisms are considered **resistant** when they survive exposure to a dose of a product that would normally kill them or stop their growth. One major concern is that microorganisms that survive exposure to a biocidal product develop resistance mechanisms that also provide protection against antibiotics used to treat human infection. This type of resistance, where the development of resistance to one type of product gives rise to resistance to another molecule is called **cross-resistance**.

In 2017, the WHO released the first ever list of “priority pathogens” that are resistant to antibiotics. The list includes 12 bacterial families that pose the greatest threat to human health. The list is drawn up to guide and promote the research and development of new antibiotics, which is part of the WHO's efforts to respond to the increasing global resistance to antimicrobials. The list specifically emphasizes the threat of Gram-negative bacteria that are resistant to many antibiotics (3). These bacteria have inherent abilities that enable them to find new ways to resist treatment, which may be spread through genetic material so that other bacteria are also resistant to drugs. The list is reproduced in Table 1. Interestingly, many bacterial species that were identified in this review for their resistance to biocidal products are also present on this list, suggesting that efforts to tackle resistance to antibiotics and biocidal substances may be combined.

Table 1 - WHO priority pathogens list

Priority 1: CRITICAL	Priority 2: HIGH	Priority 3: MEDIUM
Acinetobacter baumannii	*Enterococcus faecium*	*Streptococcus pneumoniae*
Pseudomonas aeruginosa	*Staphylococcus aureus*	*Haemophilus influenzae*
Enterobacteriaceae	*Helicobacter pylori*	*Shigella spp.*
	Campylobacter spp.	
	Salmonellae	
	Neisseria gonorrhoeae	

Since the beginning of the SARS-CoV-2 pandemic, hard surfaces and hand disinfection has been hailed as one of the most effective ways to prevent the spread of the virus. Although no data on the use of biocide is available, it is clear that it has increased dramatically, not only in the clinical space, but in public space and household settings. Interestingly, the increased use of disinfectant substances might help prevent the spread of the virus, but might also increase the occurrence and further selection of microorganisms that are resistant to biocidal products and potentially cross-resistant to antibiotics, which would greatly hinder the fight against drug-resistant microorganisms.

Scope of this report

This report aims to review the literature pertaining to the resistance of microorganisms to biocidal products belonging mostly to categories PT1 and PT2 (see Table 2**), although the development of resistance following the use of biocides that belong to other categories will be mentioned as well.**

Table 2 – Product types 1 and 2 (PT1 and PT2). Information on the other product types can be found at **https://echa.europa.eu/regulations/biocidal-products-regulation/product-types**

PT1 and PT2 belong to group 1 among 4 groups

Main group 1: **Disinfectants**

These product types exclude cleaning products that are not intended to have a biocidal effect, including washing liquids, powders and similar products.

PT1 Human hygiene

Products in this group are biocidal products used for human hygiene purposes, applied on or in contact with human skin or scalps for the primary purpose of disinfecting the skin or scalp.

PT2 Disinfectants and algaecides not intended for direct application to humans or animals

Used for the disinfection of surfaces, materials, equipment and furniture which are not used for direct contact with food or feeding stuffs. Usage areas include, *inter alia*, swimming pools, aquariums, bathing and other waters; air conditioning systems; and walls and floors in private, public, and industrial areas and in other areas for professional activities.

Used for disinfection of air, water not used for human or animal consumption, chemical toilets, waste water, hospital waste and soil.

Used as algaecides for treatment of swimming pools, aquariums and other waters and for remedial treatment of construction materials.

Used to be incorporated in textiles, tissues, masks, paints and other articles or materials with the purpose of producing treated articles with disinfecting properties.

Concerning microorganisms, the bulk of this report focuses on sporulating and non-sporulating bacteria, as these organisms are the subject of the vast majority of studies on resistance to biocides. The data available on mycobacteria, yeasts and molds were included when available. Since viruses only replicate (and mutate) within the host, resistance following disinfection with a biocidal product is highly unlikely to happen. Accordingly, no data on this subject was found in the literature.

Because of the plethora of biocidal products that are available, we decided, in consultation with the administration, to focus this report on the following active substances: alcohols (ethanol, 1-propanol, 2-propanol), aldehydes (formaldehyde and glutaraldehyde), hydrogen peroxide, peracetic acid, chlorhexidine, quaternary ammonium compounds (mostly benzalkonium chloride), chlorine releasing compounds (mostly sodium hypochlorite) and weak organic acids. Although not authorized in the EU, triclosan was also included as it constitutes an interesting case study. The risks regarding the emergence of resistance associated with other biocidal substances have also been summarized. This report focuses on the use of biocides in clinical settings, but includes occurrences of resistance following the use of biocides in other settings (household, agricultural, production), as they may also be pertinent when evaluating antimicrobial resistance in clinical settings.

Many microorganisms are intrinsically resistant to biocidal products. While it may be interesting to study these occurrences of resistance to provide guidelines for the use of biocidal products, this review focuses on the emergence of resistance following the use of biocidal products, and thus mostly on acquired and induced resistance, although interesting cases of intrinsic resistance are mentioned as well.

Concepts and difficulties surrounding microbial resistance

First, as evidenced by Munita & Arias (4), it is important to understand that resistance to antimicrobials is an ancient phenomenon that results from the interaction of different microorganisms between themselves and with the environment. Many antimicrobials are natural compounds or are derived from natural compounds; microorganisms have evolved in their native environment to overcome their lethal action and survive. This type of resistance is often called **intrinsic resistance**. Intrinsic resistance is a trait that is shared universally across a given species, is independent of previous antimicrobial exposure and is not related to horizontal gene transfer (5). The most striking example of intrinsic resistance is the double layered membrane of Gram-negative bacteria, with an outer layer of lipopolysaccharides that confer reduced permeability to the membrane and increased resistances to many antimicrobials compared to Gram-positive species. When different genes or stress responses, which are not always expressed, are induced in response to exposure to antimicrobials, the term **induced resistance** is used. An example of induced resistance is the response to acid stress, where bacteria exposed directly to an acidic solution will die quickly, but bacteria that were first exposed to a mildly acidic solution will survive the transfer to a solution to an otherwise lethal pH. This phenomenon is also known as adaptation (although adaptation may also refer to the "stepwise training" process that is explained below).

In clinical settings, where resistance to antibiotics is a pressing issue, much more attention is devoted to **acquired resistance**, which is either the result of mutations in chromosomal genes or the acquisition of external genetic determinant of resistance from other microorganisms in the environment (4). For instance, bacteria can acquire plasmids with genes encoding for resistance to multiple drugs and become multi drug-resistant (MDR). When it comes to biocidal products though, all types of resistance are relevant. Disinfecting a surface with a suboptimal concentration or quantity of biocide may lead to the survival of microorganisms that have a higher intrinsic resistance, or it may lead to the expression of stress responses and genes that enhance survival in a different condition or even in the presence of antibiotics. These surviving bacteria may then colonize other surfaces and ultimately infect humans. Similarly, disinfection of drinking water with inadequate biocides is the perfect ground for sharing genetic material between microorganisms that may lead to disinfectant and/or antibiotic resistance.

Resistance to antimicrobials **in practice** is a relative concept that has many layers of complexity. The establishment of clinical susceptibility breakpoints (susceptible, intermediate and resistant) mainly relies on the *in vitro* activity of an antimicrobial (4). While these susceptibility breakpoints are available for most antibiotics, they are usually lacking for biocidal products. Indeed, the activity of biocides is affected by several factors, including concentration, period of contact, pH, temperature, the presence of organic matter, the nature (spore, bacteria, yeast, virus) and condition (planktonic, dormant, biofilm cells) of the microorganism subjected to biocidal activity. The activity of the biocide may also be enhanced by other chemical agents such as permeabilizers (which can increase the susceptibility of Gram-negative bacteria to biocides) or efflux inhibitors. The formulation of biocidal products and condition of use should thus also be tested, in addition to the pure, active compound in the biocidal product (6).

The sensitivity of microorganisms to antimicrobials is usually measured with the **minimum inhibitory concentration (MIC)**. The MIC is the minimal concentration at which a substance will visibly prevent the growth of a microorganism. Similarly, **the minimum bactericidal concentration (MBC)** is the lowest concentration at which a substance will kill bacteria (or another microorganism). However biocides that are used as surface disinfectants or antiseptics are usually used in concentrations that are well above the MIC (6). A small increase in the MIC detected under laboratory conditions may thus have no implication on the potential of resistance in real-world conditions. Testing of the inhibitory and lethal effects, and related resistance, should be undertaken both in the laboratory and in simulated/actual condition of use, but such studies are extremely rare (6).

One method of assessing the development of resistance to antimicrobials is "stepwise training", where microorganisms are exposed to gradually increasing concentrations of a specific substance. As we show in this report, populations of microorganism with reduced susceptibility to biocides sometimes arise in these conditions, but it does not necessarily mean that the population is clinically resistant to the test substance. It can indicate a phenotypic, but not genetic, adaptation to a changing environment and stressful conditions, and susceptibility may be restored upon withdrawal of the biocide (6).

The last concept that is important to grasp is the difference between **resistance**, **tolerance** and **persistence** (reviewed by Brauner (7)). **Resistance** is typically caused by inheritable mutations, whereas **tolerance** is generally understood to be the ability of a bacterial population to survive a transient exposure to a bactericidal antimicrobial, even at concentrations that far exceed the MIC. **Tolerance** may be inherited or not. **Persistence** describes the process by which a subpopulation that is not killed by an otherwise lethal dose of antimicrobial emerges in a susceptible population. When these surviving subpopulations are grown in the presence of the same antimicrobial, the heterogeneous response is repeated, *i.e.*, the vast majority of the population is killed off, while a very small fraction survives. **Persistence** is thus not a heritable trait: descendants of the surviving subpopulation will yield the same small fraction of surviving population when exposed to the antimicrobial. A **resistant** strain will have a substantially higher MIC than a susceptible strain. A **tolerant** strain and a susceptible strain will have a similar MIC. However, the minimum duration for killing (MDK, for instance MDK_{99}, the minimum time required to kill 99% of the cells) for a **tolerant** strain will be significantly higher than the MDK_{99} for a susceptible strain. A **persistent** strain will have a similar MIC and MDK_{99} compared to a susceptible strain. However, the **persistent** strain will have a tiny fraction of its population that requires more time to kill, *i.e.*, its $MDK_{99.99}$ will be higher than that of a susceptible strain. These concepts are illustrated in Figure 1 from Brauner (7).

In practice, resistance, tolerance and persistence are all variations of the general theme of "antimicrobial resistance", as they are just different mechanisms of microorganism survival. In the long run, understand the contribution of each of these mechanisms will be primordial, as strategies used to combat resistant, tolerant or persistent microorganisms are different. However, in the literature, these concepts usually refer to antibiotics and not biocides, are often used interchangeably and sometimes the data provided in the study is not sufficient to discriminate between one term or the other. Thus, in this report, the term "resistance" will be used except if the data can be clearly attributed to the concept of tolerance or persistence.

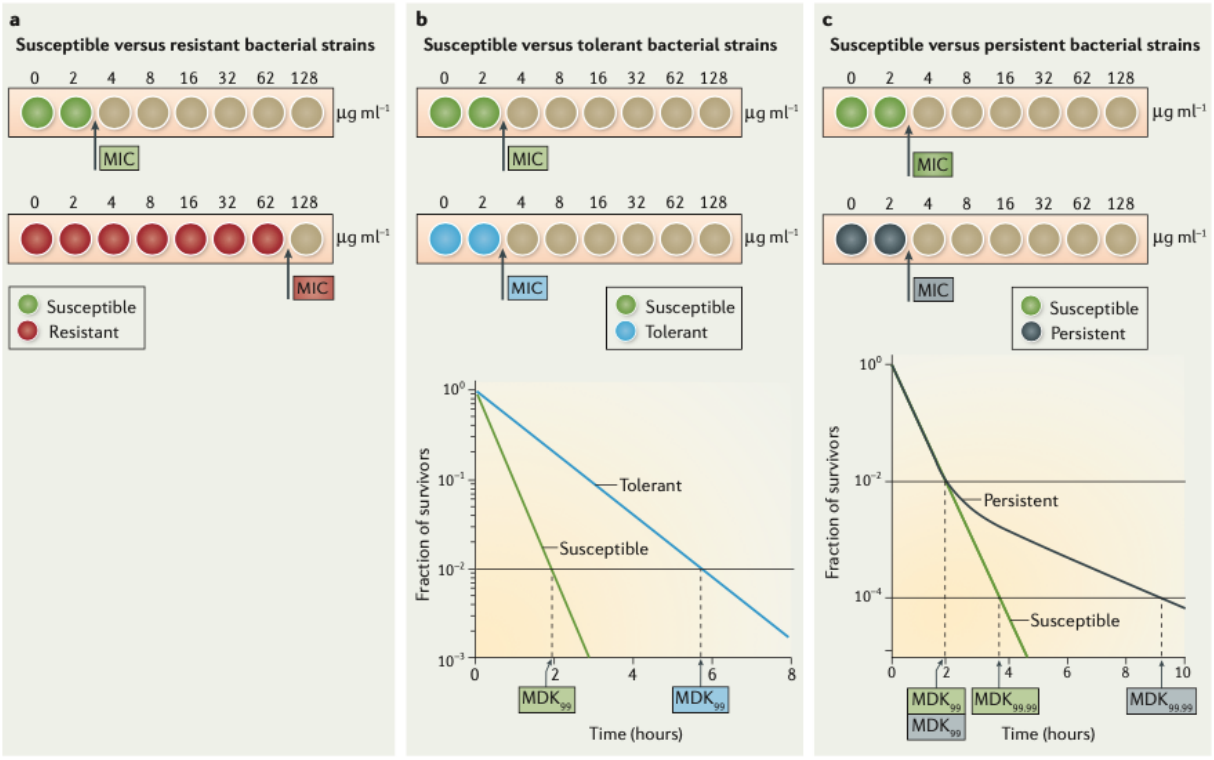

Figure 1 - Differences between resistance, tolerance and persistence.
Figure from Brauner (7)

In the next section, we summarize the major mechanisms that can contribute to microbial resistance to biocides, namely biofilm formation and efflux systems.

Mechanisms contributing to microbial resistance to biocides

Resistance to antimicrobials usually falls into 3 main categories: 1/ limiting the uptake of antimicrobials, 2/ modifying the antimicrobial target or inactivating the antimicrobial, and 3/ exporting antimicrobials outside of the cell (active efflux) (5). As evidenced in the following sections of this report, limiting the uptake of antimicrobials and exporting antimicrobials outside of the cell *via* efflux system are major determinants that are common for many classes of biocides, while modifying the antimicrobial target or inactivating the antimicrobial are much more common mechanism used to resist antibiotics. Here we will present the general concepts surrounding the mechanisms of biocide resistance.

Limiting the uptake of antimicrobials

This process is naturally carried out by the surface of the microorganism, which can be a membrane or a cell wall depending on the type of microorganism. There are however mechanisms that can influence how well this activity is performed. As mentioned earlier, Gram-negative bacteria have an LPS layer in their outer membrane that provides a barrier against a large range of molecules. For these organisms, substances may enter the cells through channels inside the membrane called “porins”. One mechanism to limit antimicrobial uptake is by either limiting the number of porins that are expressed, or changing the selectivity of the porin through mutation (5). Modifying LPS synthesis pathways is also linked to increased resistance to biocidal products (8-10).

Another major way of limiting antimicrobial uptake is through biofilm formation. Biofilms are defined as "aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface" (11). Biofilm formation occurs first through attachment to a surface and subsequent production of the extracellular matrix usually made of exopolysaccharides. Biofilms can comprise a single microbial species or multiple microbial species and can form on a range of biotic and abiotic surfaces. Mixed-species biofilms predominate in most environments, but single-species biofilms can be found in a variety of infections and on the surface of medical implants (12).

Microorganisms that are embedded in biofilms are in general much more resistant than their planktonic counterpart. For instance, bacteria within biofilms (sessile cells) are 100- to 1000-fold more resistant to antibiotics than planktonic cells (13). This increased tolerance is not yet fully understood, but is thought to be a product of both the properties of the biofilm matrix and of the slow growth that typically occurs inside biofilms (11). Indeed, the EPS may represent a diffusion barrier that impedes the penetration of antimicrobials and leads to a concentration gradient across the biofilm volume. This diffusion barrier alone does not account for the reduced susceptibility of biofilms, and some antimicrobials have been shown to diffuse through biofilms as easily as through water, but they are still quenched by components of the EPS matrix, which may involve several mechanisms, including enzymatic degradation of the antimicrobials or sacrificial reaction of the EPS (which is the case with oxidizing disinfectants) (11). Additionally, slow growth rate and dormancy have been associated with antimicrobial resistance, and biofilms contain a large number of cells that are in the stationary phase and have thus a reduced metabolic activity and an activated set of genes that are under the alternative sigma factor RpoS (which control adaptation to stationary phase) regulation. Indeed, older biofilms, that contain a larger number of cells in the stationary phase, have been shown to have higher resistance to some antibiotics such as vancomycin. Persister cells may also be prevalent in biofilm communities (11). Last, resistance to antimicrobials in biofilms may be enhanced through the increase of uptake of external genetic material by horizontal gene transfer. High cell density, increased genetic competence and accumulation of mobile genetic elements that occur in biofilms provide an ideal set of factors for efficient horizontal gene transfer, and the uptake of resistance genes (11).

How efflux pumps actively export toxic compounds out

Microorganisms may possess chromosomally or plasmid-encoded genes for efflux pump that can, as their name suggest, drive the transport of a wide variety of molecules from the inside of the cell to the outside of the cell. These pumps are either expressed constitutively or their expression can be induced in response to an external stimulus, for instance the presence of a suitable substrate. There are different types of efflux pump in microorganisms that are classified into major families depending on structure and energy source. These transporters provide different antimicrobial efflux pathways that can work in a cooperative manner or provide redundant functionality (5, 14). **The ATP-binding cassette family (ABC)** directly utilizes ATP as an energy source to drive transport. These pumps transport amino acids, drugs, ions, polysaccharides, proteins, and sugars. These pumps have fairly specific substrates, and there are very few found in clinically relevant bacteria (5). Other families are usually secondary active transporters that are powered by electrochemical energy captured in transmembrane ion gradients (14). **The major facilitator superfamily (MFS)** catalyzes transport *via* solute/cation (H^+ or Na^+) symport or solute/H^+ antiport. They are involved in the transport of anions, drugs (macrolides and tetracycline), metabolites (bile salts), and sugars. While MFS pumps have the

greatest substrate diversity as a group, individually they tend to be substrate specific. The **multidrug and toxin extrusion (MATE) family** uses a Na^+ gradient as the energy source, and efflux cationic dyes and fluoroquinolone drugs. **The small multidrug resistance (SMR) family** is energized by the proton-motive force (H^+), is hydrophobic, and effluxes mainly lipophilic cations. **The proteobacterial antimicrobial compound efflux (PACE) family** is an SMR-like family that contributes to resistance against various synthetic bactericidal agents (chlorhexidine, acriflavine, proflavine and benzalkonium). Finally, **the resistance-nodulation-cell division (RND) superfamily** catalyzes substrate efflux *via* a substrate/H^+ antiport mechanism, and is found in numerous Gram-negative bacteria. It is involved in the efflux of antimicrobials, detergents, dyes, heavy metals, solvents, and many other substrates. Many RND pumps are capable of transporting a wide range of drugs, such as the MexAB-OprM pump in *Pseudomonas aeruginosa.* The most widely studied RND pump is probably the AcrAB-TolC pump in *Escherichia coli*, which confers resistance to penicillins, chloramphenicol, macrolides, fluoroquinolones, and tetracycline (5, 14).

Efflux as a resistance mechanism in response to the use of biocidal product may be of extreme importance, as many efflux systems are also involved in high-level resistance to antibiotics. A biocidal product that increases the production of efflux systems thus represents a threat to public health.

Methodology

To carry out this review, we have used a pyramidal approach. This approach seems to us to be particularly adapted because it allows us to cover the scope of the field in question (the base of the pyramid) while highlighting the most important elements and proposing a concrete synthesis (the tip of the pyramid). Here are the different steps:

1. A very broad search in the databases (see below) was executed in order to define the framework of work, taking into account the objectives detailed in the previous section.
2. Existing literature reviews and government reports identified in the first step were used to establish an exhaustive list of research articles.
3. The list of reference articles was then expanded: recent publication and the data they present as well as key articles whose importance has been underestimated or even ignored by previous reviews and reports were uncovered. These data were analyzed and integrated into the body of knowledge.
4. The data set from the list of research articles was analyzed in detail and depth. Data concerning the emergence of resistance following the use of biocidal products was extracted and reported in the main body of this review.
5. The most relevant data was discussed and put into perspective; concrete action points, missing data and research needs were identified.

The primary database that was used for the establishment of the primary literature was PubMed. Google scholar was also used for specific searches. High-quality reviews identified through this primary search and additional reports found through the Google search engine completed the primary literature. This primary literature contained research articles and review articles from many different journals. The quality of the articles was assessed on a “one by one” basis.

For this review we decided to target the research on the different biocidal products that are in the scope of the review. This approach allowed us to be more comprehensive, more specific and more able to address the different key points of analysis that were required for this review. These key points are the following:

- The biocidal active substances (PT1 and PT2) that induce the development of resistance to antimicrobials;
- The microorganisms that become resistant to antimicrobials as a result of the use of these active substances;
- The substances (antibiotics and other antimicrobials) against which resistance (cross-resistance or not) occurs as a result of the use of biocidal active substances;
- The uses that lead to the development of antimicrobial resistance in the hospital setting;
- The mechanisms that lead to the development of microbial resistance, following use of antimicrobial products.

Of note, we understand the need to clearly identify the chemical substances that are studied in the literature. However, the CAS number of chemicals is rarely, if ever, communicated in research papers. When available, the CAS number was mentioned in this report. When not available, the supplier of the chemical product was mentioned instead. As this report is a literature review and not a quantitative risk assessment, the inability to identify the chemical substance to such a degree of precision is not an obstacle to deliver a valid opinion on antimicrobial resistance following the use of biocidal products.

Alcohols

Introduction

There are different types of alcohol that are used as biocides, but the more widely used are ethanol (CAS number 64-17-5) and the two isomers of propanol, 1-propanol (propan-1-ol) (CAS number 71-23-8) and 2-propanol (propan-2-ol) (CAS number 67-63-0), also known as isopropanol. The two isomers of propanol are authorized as biocides in the EU as PT1, PT2 and PT4, while ethanol is still under review for use as a PT1, PT2, PT4 and PT6 biocidal substance. They have rapid and broad-spectrum activity against a large range of microorganisms including bacteria and mycobacteria, fungi and viruses, although they have low activity against spores (15). Because they lack sporicidal activity, they are not recommended for sterilization, but are used widely for the disinfection of skin (in hand disinfectants and skin antiseptics for instance) and decontamination of hard surfaces. The antimicrobial activity of alcohol is considered to be significantly lower at a concentration below 50% and is optimal between 60 and 90%. The specific mode of action of alcohols as antimicrobials is still blurry, but since it is more potent when mixed with water, it is generally believed that it causes extreme membrane damage that, in addition with rapid denaturation of proteins, leads to perturbed metabolism and in the end, cell lysis (15).

Ethanol, 1-propanol and isopropanol are simple alcohols that are volatile, flammable and colorless. In living organisms, ethanol is the product of many natural biochemical pathways. According to ECHA, the European Chemical Agency, it is manufactured in and/or imported to the European Economic Area, at more than 1 000 000 tons per annum. The World Health Organization (WHO) recommends the use of alcohol based hand sanitizer in specific situations to prevent healthcare associated infections (16, 17). These alcohol based hand rubs are also recommended for the preoperative decontamination of hands for the prevention of surgical site infections (17, 18). Since 2015, the WHO has classified alcohol-based hand rubs as an “essential medicine” (17, 19). Isopropanol is also recommended by the WHO for hand sanitation, preoperative decontamination of hands and for the prevention of surgical site infections. 75% isopropanol is also considered by the WHO as an essential medicine. In contrast with ethanol and isopropanol, 1-propanol is not recommended by the WHO in such situations (19, 20).

The COVID19 pandemic has led to more than 120 000 000 contaminations worldwide and more than 2 700 000 death at the time of writing. To fight against the propagation of the virus, one of the top recommendation made by the WHO is hand washing, both using soap and water if the hands are dirty and with an alcohol-based hand rub if the hands are visibly clean (21). Demand for hand sanitizers has grown tremendously in 2020 and is visibly leading to a widespread adoption and use of the product that has yet to be fully documented. While alcohol-based sanitizers have previously been considered as safe concerning the emergence of resistance, such increase in global use may bring unforeseen consequences for human health. Here, we analyze the relevant literature regarding the emergence of resistance to alcohol in microorganisms.

Increased resistance to disinfection in biofilms

70% ethanol for 60 min has a rather poor bactericidal activity against *Acinetobacter baumannii*, *P. aeruginosa*, *Salmonella enterica* serovar. Typhimurium and *Staphylococcus aureus* (17). In 70% ethanol, biofilms of *S.* Typhimurium were viable up to 10 minutes, *E. coli* up to 30

minutes, and *Streptococcus mutans* up to 60 minutes (22). A *Serratia liquefaciens* and *Shewanella putrefaciens* dual-species biofilms showed stronger resistance to ethanol than the mono-species biofilms. Moreover, a structural observation of the biofilms indicated that the extra-cellular polymeric substances (EPS) may play an important role in the protection of dual-species biofilm. One study showed that the surface of *Bacillus subtilis* biofilms remains nonwetting against up to 80% ethanol (as well as 50% methanol and 50% isopropanol). They show that this property limits the penetration of antimicrobial liquids into the biofilm, severely compromising their efficacy (23).

Isopropanol also has low activity against bacteria in biofilm, with less than 5-$\log_{10}$ reduction in up to 60 min of treatment in the majority of studies (17).

Emergence of resistance

A previous report by the Norwegian Scientific Committee for Food Safety published in 2016 found no occurrence of resistance to alcohol-based biocides in the literature (24). It is well known however, that some microorganisms can develop some degree of resistance to low doses of ethanol (below 25%) (25, 26). It was also shown that low concentrations of alcohol (around 2%) leads to enhanced growth of different *Acinetobacter* species (27) and that low concentrations of commercial alcohol hand rubs (1%) enhanced growth of multidrug resistant strains of *A. baumannii* (28).

The maximum ethanol resistance for *Saccharomyces cerevisiae* has been demonstrated to be 25% (29). *S. cerevisiae* aBR10 cells were able to develop resistance to lethal ethanol concentrations (14%), by preexposure to a sublethal ethanol stress (8%) (30). When cells of *Listeria monocytogenes* were adapted to a sublethal dose of ethanol (5%) for 1h, they were significantly more resistant to killing by a normally lethal dose of ethanol (17.5%) (31). Similarly, cells of *Pseudomonas* sp. DJ-12 that were acclimated to 5% ethanol for 10 min had significantly increased resistance to killing by 10% ethanol (32).

Acclimation of *E. coli* (IS elements-free *E. coli* strain MDS42) to increasing concentrations of isopropanol (from 0 to 500mM) for 24 days led to a strain that was able to grow in 2.7% isopropanol (33). No MIC values were measured and the stability of the resistance to isopropanol is unknown. The strain with increased isopropanol resistance was also slightly more resistant to other alcohols (ethanol, n-propanol, n-butanol, isobutanol, and n-pentanol), indicating that the mechanism of resistance is similar for all types of alcohols (33). An isopropanol-resistant *Sphingobacterium mizutae* isolated from an oil-soil mixture was shown to be able to multiply in isopropanol solutions up to 3.8%, indicating that slight alcohol resistance can arise in specific environmental niches (34).

More recently, it was reported that hospital strains of *Enterococcus faecium* displayed increasing resistance to handwash alcohol (35). They found that *E. faecium* isolated after 2010 were 10-fold more resistant to isopropanol killing than older isolates (as old as 1997). These alcohol-resistant isolates were more resistant to standard 70% isopropanol surface disinfection than their alcohol-sensitive counterparts, leading to greater mouse gut colonization in their infection model (35). However, while isopropanol at 23% was ineffective against these isopropanol-resistant isolates, subsequent studies by other teams confirmed that isopropanol at concentrations as low as 60% was effective in killing alcohol-resistant *E. faecium* but that the volume of solution used to disinfect surfaces was crucial for efficient killing (36, 37). A smaller 2019 field study found no increase in the isopropanol MIC of clinical isolates before and after the systematized use of alcohol for hand antisepsis, and did not find a single isolate with an

MIC higher than 11.5% (38). In conclusion, results for resistance on *E. faecium* vary from one study to another.

Different clinical isolates of *Corynebacterium striatum* showed resistance to 70% ethanol if exposure was below 1 min. Interestingly, the presence of 2% bovine serum albumin increased bacterial survival to up to 30 min of contact time, indicating that the presence of biological matter may increase resistance to alcohols (39). Out of 47 *A. baumannii* clinical isolates derived from the blood, sputum or swab samples of patients, one had a MIC of 22.5% against ethanol (8 had a MIC of 15% and the rest had 7.5%) (40).

It is also worth to note that in a high viscosity medium such as artificial mucus or mucus from infected patients (sputum), alcohol diffusion time increases dramatically, leading to increased resistance of influenza A virus (PR8, A/Puerto Rico/8/1934 (H1N1)) and *E. coli* (K12 NCTC 10538) against killing by alcohol-based disinfectants such as 80% ethanol, 70% isopropanol and 60% 1-propanol, which may lead to incomplete decontamination of soiled hands and surfaces (41, 42).

Mechanism of resistance

To the best of our knowledge, no specific resistance mechanisms such as plasmids, efflux pumps or resistance genes have been described to explain bacterial or fungal resistance to simple alcohols (17). Isopropanol-resistant *E. faecium* isolates mostly accumulated mutations in genes related to metabolism and carbon uptake (35). In a strain engineered to be able to grow in 2.7% isopropanol, five mutations (*relA*, *marC*, *proQ*, *yfgO*, and *rraA*) were found to be responsible for the increased resistance to isopropanol. The expression levels of genes involved in the biosynthesis pathways of some amino acids, iron homeostasis, and energy metabolisms were changed in the resistant strain, which suggests that these gene functions are involved in isopropanol resistance (33). The presence of single or multiple disinfectant resistance genes (*qacA*, *qacDE*, *qacE*, *acrA*) might be correlated with a higher ethanol MIC value (no resistance genes: 0.0004% ; all four resistance genes: 0.0064 %) (44). The relevance of these data is pretty low considering the abysmally low MIC value compared to the concentration of ethanol that is used in practice (which is more than 10 000-fold the MIC found in this paper).

In *B. subtilis* cells, the transfer of the mobile genetic element Tn916, a conjugative transposon and the prototype of a large family of related elements, was increased 5-fold by exposure to 4% ethanol for up to 2h, which may also result in a transfer of Tn916-like elements and any resistance genes they contain (17, 43).

Conclusion

In conclusion, disinfection with alcohol-based products remains an extremely effective way of killing microorganisms. To the best of our knowledge, and despite the many years of use of alcohols as disinfectants, there have been **no reports on the emergence of resistance** when using appropriate concentrations of product, although biofilms have increase resistance towards disinfection by alcohol. A small increase in resistance was observed for *S. cerevisiae*, *L. monocytogenes*, *E. coli*, *S. mizutae*, *C. striatum*, and *A. baumannii.* **No cross-resistance** with other biocidal products or antibiotics has been reported yet. That being said, the emergence of clinically-relevant *Enterococcus* strains that are resistant to increasing concentrations of alcohol (up to 23%) highlights the need for vigilance. Alcohol-based disinfection efficacy remains very

much defined by physical constrains (size of the area to be disinfected, presence of organic matter, …) and **great care should be taken to ensure that correct disinfection procedures are followed, with focus on the volume of disinfectant used and appropriate disinfection timing so that the effective concentration of alcohol reaching the microorganism is attained.** Since the Covid-19 crisis, use of hand rubs containing alcohol has exploded, and increased use leads to more opportunity for misuse. For instance, clinically relevant strains could acclimate to low doses of alcohol and be disseminated through people that use low quality hand rubs, low quantity of hand rubs, or that do not rub for the recommended amount of time, although we consider that scenario unlikely.

Aldehydes

Introduction

Formaldehyde (CAS number 50-00-0) is a mono-aldehyde approved as PT2 and PT3 in the EU. Its clinical use is generally as a disinfectant and sterilant in liquid or in combination with low-temperature steam. Formaldehyde is bactericidal, sporicidal, and virucidal, but it works more slowly than glutaraldehyde. Formaldehyde is an extremely reactive chemical that interacts with proteins, DNA, and RNA *in vitro* (15).

Glutaraldehyde (Glutaral, CAS number 111-30-8) is a dialdehyde approved as PT2, PT3, PT4, PT6, PT11, PT12 and not approved as PT1 and PT13 in the EU. Glutaraldehyde at 2% can be found as a disinfectant in the WHO model list of essential medicines (19, 45). It is used as a disinfectant and sterilant for low-temperature surface disinfection and sterilization of endoscopes and surgical equipment. It is also used in the veterinary field, in poultry and pig farms, and for machinery and food processing surface disinfection (15, 45). Glutaraldehyde has a broad spectrum of activity against bacteria and their spores, fungi and viruses, although the mechanism involved for killing seems to be different for each organism. In bacterial spores, low concentrations inhibit germination while high concentrations are sporicidal, probably as a consequence of strong interaction with outer cell layers. In mycobacteria the action is unclear, but probably involves interactions with the mycobacterial cell wall. There is a strong association of glutaraldehyde with outer layers of Gram-positive and Gram-negative bacteria associated with cross-linking of amino groups in protein and inhibition of transport processes into the cell. In fungi, the cell wall appears to be a primary target site, with postulated interaction with chitin. The actual mechanism of killing viruses is unknown but involve protein-DNA cross-links and capsid changes (15).

Ortho-phthalaldehyde is a newer type of aldehyde disinfectant (not in the EU review program) that has potent bactericidal and sporicidal activity and has been suggested as a replacement for glutaraldehyde in endoscope disinfection. Ortho-phthalaldehyde is an aromatic compound with two aldehyde groups. The mechanism of action of this biocide seems to be similar to that of glutaraldehyde (15).

Finally, glyoxal, a small compound with two aldehyde groups, is approved as PT2, PT3 and PT4. No relevant data is available on the resistance to this compound when used as a biocide.

Increased resistance to disinfection in biofilms

The efficacy of aldehyde-based disinfectants seems impaired when used to eradicate bacteria in biofilms. While glutaraldehyde (Fisher-Scientific, USA) in concentration below 0.2% was able to eradicate planktonic cultures of *A. baumannii, Burkholderia cepacia, Enterococcus faecalis, E. faecium,* methicillin resistant *S. aureus* (MRSA), *Staphylococcus epidermidis, P. aeruginosa, Stenotrophomonas maltophilia,* and *E. coli* (ATCC 25922), much higher concentrations were required to kill 85% of viable cells in biofilms of these species (46). *P. aeruginosa* embedded in artificial biofilm were 34-fold more resistant to glutaraldehyde than planktonic cells. Increasing the concentration of the biocide increased bacterial killing more in the biofilm than in a suspension culture (47), indicating that biofilm cultures are not as easily saturated as suspension cultures, and that biofilm may need to be disinfected with higher concentrations of biocidal products. Viable cells of *Bacteroides fragilis* were recovered from biofilms after 30 min and of *S. mutans* and *Salmonella* Typhimurium after 60 min of contact with 2.4% glutaraldehyde (Vetec Quimica Fina Ltda) (48). For the disinfection of endoscope

channels, 2% glutaraldehyde (Steranios, LECTUS S.A., Buenos Aires, Argentina) was effective in 20 min and yielded negative cultures after disinfection when the channels were allowed to build *S. aureus* (ATCC 29213), *P. aeruginosa* (ATCC 27853) or *Mycobacterium abscessus* subsp. *bolletii* biofilms over 5 days. However, viable cells remained after the disinfection process. However, these cells were unable to initiate new cultures. More viable cells were recovered after disinfection with glutaraldehyde than after disinfection with peracetic acid or ortho-phthalaldehyde (49). Different clinical isolates of *C. striatum* survived treatment with 2% glutaraldehyde for 30 min when they formed mature biofilm on different surfaces (39).

Emergence of resistance

Increased resistance or tolerance to aldehyde-based disinfectants has been described in various bacterial species, including *E. coli, P. aeruginosa* and *P. fluorescens, Helicobacter pilori,* spores of *Bacillus* and *Clostridium.* The largest group of aldehyde-resistant bacteria, responsible for multiple outbreaks worldwide (50) is composed of members of the *Mycobacterium* genus. Here we detail the relevant literature.

A formaldehyde-resistant strain of *E. coli* (VU3695) was isolated from patients and contaminated disinfection solution (51). It was found to harbor a chromosomal copy of *adhC*, a glutathione-dependent aldehyde dehydrogenase and a plasmid copy of the same gene that is actively expressed and confers resistance to exogenous formaldehyde (52). No record of this strain could be found in the literature after 2004.

Glutaraldehyde-resistant strains of *P. aeruginosa* were isolated from samples obtained from endoscopes during routine surveillance. The glutaraldehyde-based disinfectant showed no activity against the 2 isolated strains using the recommended concentration in standard conditions. The strains were linked to 6 patients with lower respiratory tract and bloodstream infections (53, 54). The authors could not decipher the mechanism of resistance but postulated that biofilm formation in the old decontamination apparatus could have been a factor (53). A later study found later that genetic mechanisms were involved in glutaraldehyde resistance in biofilms of *Pseudomonas fluorescens* and *P. aeruginosa* (55). Their RNA-seq data showed that efflux pumps and phosphonate degradation, lipid biosynthesis, and polyamine biosynthesis metabolic pathways were induced upon glutaraldehyde exposure. On the other hand, chemical inhibition of efflux pumps potentiates glutaraldehyde activity, which suggests that efflux activity contributes to glutaraldehyde resistance. They also noted the induction of known modulators of biofilm formation, including phosphonate degradation, lipid biosynthesis, and poly-amine biosynthesis, which may contribute to biofilm resistance and resilience (55).

Several clinical isolates of *H. pilori* were shown to have higher resistance to glutaraldehyde. This resistance phenotype was associated with increased expression of genes involved in LPS biogenesis (9, 56). There was no other mention in the literature of *Helicobacter* being resistant to aldehyde-based disinfection after 2009. Different clinical isolates of *C. striatum* expressed different resistance pattern to 2% glutaraldehyde, with one isolate surviving up to 30 min in the presence of the biocide.

Spores of *B. subtilis*, *Bacillus anthracis* and *Clostridium sporogenes* displayed differential viabilities when exposed to commercial aldehyde-based disinfectant (CIDEX), with *B. subtilis* requiring more than 200 min to achieve a 6-$\log_{10}$ reduction, compared to around 5 min for *B. anthracis* and 23 min for *C. sporogenes*. Heat-shocking the spores after disinfectant treatment had little to no effect on the efficacy of these treatments (57). There was no information on potential mechanisms of resistance, although unrelated laboratory tests showed

that spores of *B. subtilis* carrying mutations associated with rifampicin resistance on the RNA polymerase β-subunit *rpoB* gene are slightly more resistant to formaldehyde and glutaraldehyde than non-mutant strains (58).

Perhaps the most occurrences of resistance to aldehyde-based disinfectant are reported in the *Mycobacterium* genus. *Mycobacterium chelonae* was reported to be able to develop resistance to glutaraldehyde as early as 1993 (59). Since then, many clinical isolates from the *Mycobacterium* genus were shown to be resistant to killing by glutaraldehyde (60-64). Between 2004 and 2008, in Brazil, an outbreak of *M. abscessus* subsp. *massiliense* infections (>2,000 possible cases) following video-assisted surgery was caused by a single highly virulent clone displaying high-level resistance to glutaraldehyde (64). In the US, in a panel of 117 clinical isolates of rapidly growing mycobacteria isolated between 1994 and 2008 in a single hospital, 6 isolates belonging to the emerging *M. abscessus* group displayed significant resistance to glutaraldehyde and ortho-phthalaldehyde (61). In the US, automated endoscope reprocessors at 3 different clinical sites were sampled post-disinfection, and bacterial contamination was found in all instances. Species included *Mycobacterium* and *Methylobacterium.* The isolated bacteria were either sensitive to aldehyde-based disinfectants, suggesting that they may have formed biofilms in the apparatus, or resistant. The resistant isolates included *M. gordonae, M. chelonae, M. abscessus/chelonae* and *M. avium,* and all isolates were sensitive to oxidizing agents (62). A later study showed that different commercially available products based on glutaraldehyde or ortho-phthalaldehyde had variable efficacy against glutaraldehyde resistant strains of *Mycobacterium* isolates, but that peracetic acid- and hydrogen peroxide-based disinfectants efficiently killed all of the *Mycobacterium* isolates (60).

Formaldehyde-resistant *E. coli* and *Halomonas* spp. strains were also resistant to high concentrations of glutaraldehyde and acetaldehyde (65). Similarly, a glutaraldehyde-resistant *B. cepacia* isolate also exhibited cross-resistance to formaldehyde (45, 66).

Mechanisms of resistance

In biofilms of *P. aeruginosa* and *P. fluorescens,* efflux pumps may contribute to glutaraldehyde resistance. RNA-seq data show that efflux pumps and phosphonate degradation, lipid biosynthesis, and polyamine biosynthesis metabolic pathways were induced upon glutaraldehyde exposure and chemical inhibition of efflux pumps potentiates glutaraldehyde activity, suggesting that efflux activity contributes to glutaraldehyde resistance (55).

In some bacteria, resistance to aldehyde-based disinfectants seems to depend on the composition of the membrane. To gain more insights into formaldehyde resistance, two formaldehyde-resistant strains, *E. coli* VU3695 and *Halomonas* sp. MAC (DSM 7328), were studied. The presence of high levels of formaldehyde dehydrogenase activity alone did not confer resistance to high formaldehyde concentrations. These formaldehyde-resistant strains also proved to be resistant to high concentrations of acetaldehyde and glutaraldehyde, which are not oxidized by formaldehyde dehydrogenase. However, treatment with sublethal concentrations of EDTA (which destabilizes the outer membrane) rendered the resistant strains highly sensitive to formaldehyde without affecting the activity of formaldehyde dehydrogenase. The membrane of the resistant strains has altered composition compared to sensitive strains (65). In *H. pylori*, the expression levels of *imp/ostA* (*lptD* in *E. coli)*, a component of the lipopolysaccharide assembly machinery, and *msbA*, the lipopolysaccharide flippase, were correlated with glutaraldehyde resistance in clinical isolates after glutaraldehyde treatment (9, 56).

The mechanism of resistance of *Mycobacterium* to aldehyde-based disinfectants is still unknown, but there is evidence that defects in porin expression (*msp* genes) increase the resistance of *Mycobacterium smegmatis* and *M. chelonae* to formaldehyde, glutaraldehyde and ortho-phthalaldehyde (67, 68). Because defects in porin activity also increased the resistance of *M. chelonae* to drugs (rifampicin, vancomycin, ciprofloxacin, clarithromycin, erythromycin, linezolid and tetracycline), it is not impossible that the widespread use of glutaraldehyde and ortho-phthalaldehyde in clinical settings may select for drug-resistant bacteria (68). In glutaraldehyde-resistant *M. chelonae*, efflux pumps were not found to play a role in resistance to the biocide (69).

Conclusion

In conclusion, increased resistance or tolerance to aldehyde-based disinfectants has been described in various bacterial species, including *E. coli, P. aeruginosa* and *P. fluorescens, H. pilori,* spores of *Bacillus* and *Clostridium.* However, most of these reports seem to emerge from lab experiments or a very isolated instrument contamination in the clinical setting, usually blamed on the emergence of biofilm on old medical decontamination devices. Indeed, biofilms of many bacterial species are much more resistant to disinfection with aldehyde-based products. **More concerning is the emergence of resistance amongst the *Mycobacterium* genus, which seem to be causing more and more outbreaks around the globe after colonization of decontaminating devices**. Cross-resistance to other aldehyde-based compounds has been observed, but there are no reports yet of cross-resistance against other biocidal products or antibiotics. The resistance mechanism is not elucidated yet, but may involve reduced efflux systems and differential porin expression, which may in turn lead to increased resistance to some antibiotics, suggesting that the development of cross-resistance in these strains is a possibility. **Since some of these isolates seem to be resistant to formaldehyde, glutaraldehyde and ortho-phthalaldehyde, but all are usually still sensitive to oxidizing agents (such as peracetic acid), it may be wise to generalize the use of such oxidizing agent for decontamination of medical apparatus instead of aldehyde-based disinfectants in the future**.

Hydrogen Peroxide

Introduction

Hydrogen peroxide (H_2O_2, CAS number 7722-84-1) is a widely used biocide for disinfection, sterilization, and antisepsis approved as a PT1, PT2, PT3, PT4, PT5 and PT6 product in the EU. It is a clear, colorless liquid that is commercially available as an aqueous solution in various concentrations ranging from 3-90% of hydrogen peroxide. Commercial hydrogen peroxide solutions are usually stabilized to prevent or slow down its decomposition. It is considered to be environmentally friendly because it degrades rapidly into harmless water and oxygen. It is used for the disinfection of human skin, hospital items such as endoscopes, and hard surfaces in healthcare and veterinary institutions. It can be used as a solution directly in contact with the surface to be disinfected (at around 5%), or vaporized into the air, leading to a concentration around 250-400 ppm in air, equivalent to 0.025-0.04% of H_2O_2 (15, 70).

H_2O_2 demonstrates broad-spectrum efficacy against viruses, bacteria, yeasts, and bacterial spores. Hydrogen peroxide between 0.5 and 10% was found to be mostly bactericidal within 30 min. In general, greater activity is seen against Gram-positive than Gram-negative bacteria. Sporicidal activity requires higher concentrations of H_2O_2 (10 to 30%) and longer contact times. H_2O_2 has fungistatic activity against *Candida albicans, C. glabrata, C. krusei and C. tropicalis* with MIC values generally ranging from 0.00001 to 0.00045%. However, the fungicidal activity of 3% H_2O_2 is generally poor at exposure times below 10 min. Even longer exposure times (up to 6h) do not usually yield more than a 4-$\log_{10}$ reduction (15, 70).

H_2O_2 acts as an oxidant by producing hydroxyl free radicals (•OH) via the Fenton reaction; hydroxyl radicals attack essential cell components, including lipids, proteins, and DNA. It has been proposed that exposed sulfhydryl groups and double bonds are particularly targeted (15, 70).

It is important to note here that, as is the case with alcohol, ROS and ROS-generating chemicals are normal bacterial metabolites that are constantly generated during bacterial aerobic respiration. A fine-tuned control between the formation and detoxification of ROS leads to a balanced redox status of the cell. However, when this equilibrium is perturbed, elevated ROS levels can exceed cellular tolerance and lead to oxidative stress. This stress can damage macromolecules, including proteins, DNA, and lipids, eventually resulting in cell death. To protect cells from the harmfulness of ROS, aerobic bacteria contain enzymes (catalases, superoxide dismutases (SOD), glutathione peroxidases and peroxiredoxins) that can detoxify ROS, and prevent premature death (71, 72).

While there are mechanisms in place to resist oxidative stress, it is unclear how these mechanisms play a role in resisting disinfection with oxidative agents, as the literature on this subject is sparse. However, there are some interesting data that are shared in this section.

Increased resistance to disinfection in biofilms

Most studies show that hydrogen peroxide is less effective against bacterial cells in biofilms. While a bactericidal activity against planktonic cells is mostly achieved with 0.5% hydrogen peroxide in 30 min, a 4.0-$\log_{10}$ reduction in biofilm is mostly not achieved using 2 or 3% hydrogen peroxide in 30 min (70). Single-species biofilms of several clinical isolates of *Acinetobacter* spp.*, Klebsiella pneumoniae* and *P. aeruginosa* showed up to 266-fold less sensitivity to H_2O_2 compared to planktonic cultures. All tested species were similarly

susceptible to low concentrations of H_2O_2 (0.0017 to 0.07%) in planktonic cultures (73). Biofilms of *Burkholderia cenocepacia* were also shown to be resistant to H_2O_2 treatment at different concentrations (0.3% - 3%), while planktonic cells were sensitive (74). This effect of resistance through biofilm formation may be even higher in the wild, as mixed communities biofilms show a markedly increased (min 4-fold) resistance to exposure to hydrogen peroxide compared to single-species biofilms (75). Treating drinking water biofilm with 3% hydrogen peroxide resulted in an immense population shift, indicating that disinfection of drinking water might select for persisters and tolerant microorganisms which can live on the residuals of the dead biofilm cells (76). Of note, hydrogen peroxide (0.5 to 7.2%) used for between 1 and 5 min had an overall better disinfection efficacy than two quaternary ammonium compounds against biofilms of *S. aureus* (ATCC-6538) and *P. aeruginosa* (ATCC-15442) (77).

The activity of H_2O_2 against yeast cells in biofilm is also lower compared to planktonic cells. One study shows that strains of *C. albicans*, *C. parapsilosis* and *C. glabrata* required 2-8 times higher concentrations of H_2O_2 for efficacy against biofilms than for efficacy against planktonic controls (78). *Candida auris* biofilms also displayed increased resistance to hydrogen peroxide compared with planktonic cells, and early biofilm were also more sensitive to hydrogen peroxide than mature biofilms (79).

Emergence of resistance

Development of resistance was observed in *E. coli*. Pre-treatment of *E. coli* with a low dose (0.0002%) of H_2O_2 increases the survival upon subsequent exposure to an otherwise lethal dose (0.1%) in a process called ‘priming’ (80). It was also shown that the 'priming' response had a protective role from lethal mutagenesis. Bacteria that were primed evolved H_2O_2 resistance faster and to a higher level (up to 0.05%) (81).

Development of resistance was also observed when *M. tuberculosis* was exposed to increasing concentrations of hydrogen peroxide. A resistant mutant with 80-fold increase in the MIC (0.01%) compared to a wild-type strain (0.00013%) was isolated. This mutant was shown to have increased *katG* (a catalase) expression, and an increased growth rate in nutrient-depleted conditions or in macrophages (82).

Campylobacter jejuni is usually oxygen-sensitive, but hyper-aerotolerant *C. jejuni* strains were isolated from retail raw chicken meat. These strains exhibited significantly increased activities of catalase and superoxide dismutase (SOD), compared to the oxygen-sensitive strains and were more resistant to oxidants, such as hydrogen peroxide (0.0034 – 0.0068%) and peracetic acid (0.075%, see next section) compared to the oxygen-sensitive strains (83, 84).

Exposure of biofilms cells to sublethal concentration of hydrogen peroxide (0.44%) enhanced the biofilm forming ability of *Salmonella* Enteritidis NCTC 13349 (85).

Cross-resistance to other biocides was shown in one study, where pretreatment of *E. coli* K12 with 0.1% hydrogen peroxide (Sigma) led to increased resistance to 0.2% hypochlorous acid (86).

Exposure of the yeast *S. cerevisiae* (strain CY4) to a sub-inhibitory concentration of H_2O_2 (0.007%) for 1h led to a reduced susceptibility against an otherwise lethal dose of 0.07% hydrogen peroxide (87). Preadaptation with 0.0017% hydrogen peroxide led to cross-resistance to 20% ethanol with 100% survival of the yeast strain (88).

Interesting cases

Resistance to hydrogen peroxide may be of less importance than to other known biocidal substances. Indeed, when diverse food-grade biocidal formulations containing triclosan, chlorhexidine, benzalkonium chloride or hydrogen peroxide were used to select for biocide-resistant *Salmonella* strains, resistance to triclosan, chlorhexidine and benzalkonium chloride emerged, but not to hydrogen peroxide (89).

Commercially available bioindicators, most frequently *Geobacillus stearothermophilus* spores, are often used to assess the efficacy of disinfection. One study aimed to investigate the resistance of methicillin-resistant *S. aureus* (MRSA) to vaporized hydrogen peroxide (which is used in the decontamination of hospital isolation rooms) in comparison with these commercially available biological indicators. They found that the recovery of MRSA was between 1.5 and 3.5-$\log_{10}$ higher than the recovery of *G. stearothermophilus* spores. The authors attribute this greater resistance to the potential production of catalase, resulting in a reduction of the effectiveness of vaporized hydrogen vapor. These findings highlight that the reduction achieved with a commercially available biological indicator cannot always be extrapolated to other micro-organisms, and that different species have different susceptibilities to vaporized hydrogen peroxide (90).

Hydrogen peroxide is also used in pre-wetted disinfectant wipes. A brief study found that while these wipes had sporicidal activity and that hydrogen peroxide wipes were more sporicidal than wipes with quaternary ammonium compounds, all disinfectant wipes transferred *Clostridioides difficile* spores from contaminated to otherwise previously uncontaminated surfaces (91). This highlights once again that the method of application is as important as the type of disinfectant used.

Hydrogen peroxide as a mist or in liquid state is also efficient against food-related molds such as *Alternaria alternata*, *Aspergillus flavus*, *Geotrichum candidum*, *Mucor plumbeus*, *Paecilomyces variotii*, and *Penicillium solitum,* although some species seem to be more resistant than others (92).

Mechanism of resistance

As mentioned earlier, hydrogen peroxide is degraded by peroxidases and catalases into water and molecular oxygen. These enzymes are naturally present in bacteria and yeasts and are involved in the resistance against hydrogen peroxide. The main genes involved are *katA, katE,* and *katG,* which are directly involved in the resistance mechanism, and *oxyR*, which is a transcription regulator that mediates the response to hydrogen peroxide. Here are specific examples of resistance mechanisms.

A strain of *Serratia* (*Serratia* sp. LCN16) isolated from a plant parasitic nematode was found to be highly resistant to oxidative stress. This strain was able to grow in 3% H_2O_2. This resistance phenotype was found to require the presence of the H_2O_2 transcriptional factor *oxyR*; and the H_2O_2-targeting enzyme, catalase *katA* (93). It is unknown how this strain developed this resistant phenotype.

In a strain of *E. coli* that was adapted to hydrogen peroxide, protection against the biocide was shown to be provided by long-lived proteins (such as KatA and AhpF) that, upon pre-exposure, remained at a high level for several generations. The mutations that increased resistance to H_2O_2 did not occur in known ROS scavenger encoding genes. The type and number of mutations

indicate that scavenger systems against oxidative stress are optimally evolved since no mutations directly affecting these systems were found under H_2O_2 stress selective pressure. Instead, for example, there were mutations of the flagella regulator or in the *fim* operon. The authors hypothesize that H_2O_2 could thereby stimulate the early stages of biofilm formation, thus providing additional protection against ROS (81).

In *Salmonella* Typhimurium, SlyA is a transcription factor that regulates the expression of genes involved in virulence (*sopD, sopE2, hilA)* and central metabolism (*kgtP, glpA, fruK)* in response to hydrogen peroxide and sodium hypochlorite (94). Incomplete disinfection with hydrogen peroxide might thus potentiate bacteria to become more virulent, although that hypothesis has not been tested yet.

Conclusion

Microorganisms in biofilms are much more resistant to decontamination by hydrogen peroxide. There are a few reports on the emergence of higher resistance towards hydrogen peroxide following exposure to the biocide in *S. cerevisiae*, *E. coli, M. tuberculosis* and *C. jejuni.* Low level cross-resistance to 0.2% hypochlorous acid has been observed in *E. coli* and to 20% ethanol in *S. cerevisiae*. **Nevertheless, these reports are few, and none of them are clinically relevant yet.** The mechanisms of resistance are not fully elucidated yet, but may involve specific enzymes such as catalases, superoxide dismutases, glutathione peroxidases and peroxiredoxins that target ROS and their toxic by-products, as well as a pleitropic response that is mediated by global regulators such as OxyR or SlyA.

Hydrogen peroxide is an efficient biocide with a non-specific mode of action that readily decompose into non-toxic product in the environment. All **these features make peroxygen compounds such as hydrogen peroxide very attractive disinfectants.**

Peracetic Acid

Introduction

Peracetic acid (CAS number 79-21-0) is an organic peroxide and a colorless liquid with a characteristic acrid odor reminiscent of acetic acid. It is approved as a PT1, PT2, PT3, PT4, PT5, PT6, PT11 and PT12 product in the EU. Peracetic acid is obtained by reacting hydrogen peroxide with acetic acid in an aqueous solution. In this process, peracetic acid is not obtained as a pure substance but in the form of an aqueous solution containing peracetic acid, acetic acid, hydrogen peroxide and water (95). Peracetic acid is usually considered a more potent biocide than hydrogen peroxide, being sporicidal, bactericidal, virucidal, and fungicidal even at low concentrations (0.3%). Dry-fogging a mixture of hydrogen peroxide and peracetic acid is an efficient way to inactivate non-enveloped and enveloped viruses (including SARS-CoV-2), mycobacteria and bacterial spores. However some species are more resistant than others (notably *Mycobacterium senegalense*) (96). Adeno-associated virus are inactivated by peracetic acid, sodium hypochlorite and potassium peroxymonosulfate, but not by hydrogen peroxide and ethanol (97).

As is the case with hydrogen peroxide, peracetic acid is considered to be environmentally friendly as it decomposes into safe by-products (acetic acid and oxygen). Compared to hydrogen peroxide, peracetic acid has the added advantages of being free from decomposition by peroxidases, and remaining active in the presence of organic loads. Its acts in a similar manner as H_2O_2, probably by denaturing proteins and enzymes and increasing cell wall permeability by oxidizing sulfhydryl bonds. Its main application is as a low-temperature liquid sterilant for medical devices, flexible scopes, and hemodialyzers, but it is also used as an environmental surface sterilant (15).

Increased resistance to disinfection in biofilms

While peracetic acid has great efficacy with planktonic cells, killing bacterial cells in biofilms is more difficult. Increased resistance to peracetic acid for bacteria embedded in biofilms has been reported for multiple species. In most cases, either longer incubation time or increased concentration of the product are necessary for efficient killing. Increased resistance to killing in biofilms compared to planktonic cells has been shown in *E. coli, S. aureus, S. epidermidis, Klebsiella* spp.*, L. monocytogenes, P. aeruginosa* and *Salmonella* spp. (98-107)

Mixed species biofilm of *L. monocytogenes* and *Lactobacillus plantarum* were also more resistant to peracetic acid (Sigma-Aldrich) than single-species biofilms (108).

However, biofilms of *P. aeruginosa* were able to survive 5 min treatment with 2000 ppm peracetic acid (38 - 40%; Merck), which is the working concentration in some washers/disinfectors. One study showed that in the presence of organic matter, bacterial biofilms of *S. aureus, S. epidermidis, E. coli* and *Klebsiella* spp. can tolerate extremely high concentrations of oxidizing agents, including peracetic acid. Peracetic acid still had a robust effect on water biofilm (with no extra organic matter), indicating that this chemical might be efficient at killing biofilms on clean surfaces, but not on dirty ones (98).

While the efficacy of peracetic acid was reduced in biofilm, in many studies it remains the most efficient of the biocide tested to eradicate biofilms, including compared to substances such as cetrimide, chloroxylenol, phenoxyethanol (99) and benzalkonium chloride (101). 24-hour biofilms of *Pseudomonas marginalis* were more resistant than planktonic cells to sodium

hypochlorite, Bardac 2050 (a mixture of quaternary ammonium compounds) and hydrogen peroxide, but not to peracetic acid (in a mixture of 35.5% peracetic acid, 39.5% acetic acid, 6.8% hydrogen peroxide and 1% sulfuric acid) (109), suggesting that this biocide may be more indicated for the removal of biofilms than other options.

Emergence of resistance

The risk of the development of resistance is regarded to be very low due to the low specificity of reactions of peracetic acid (95). The expected low occurrence of resistance during peracetic acid disinfection has been confirmed in real-world cases. In one study, treatment of wastewater effluents with peracetic acid (PanReact AppliChem) led to a significant reduction in the percentage of ampicillin-resistant *E. coli*, compared to an untreated wastewater control (110). Another study found that treating wastewater effluents sample with either UV or peracetic acid leads to a similar reduction (around 50%) of the proportion of uropathogenic *E. coli* in the total bacterial population. Furthermore, treatment with peracetic acid (Solvay Interox) was associated with a substantial reduction of the detection of antimicrobial resistance genes in wastewater, while treatment with UV was not (111). Other studies focusing on wastewater disinfection found no statistically significant decrease of antimicrobial resistance genes, but no increase either (112, 113).

Nevertheless, there are studies showing that some level of tolerance can result from exposure to peracetic acid. *S.* Typhimurium LT2 cells that were treated with 0.0015% peracetic acid (Bactipal D, Seppic SA, Paris, France) were viable but nonculturable and retained virulence as demonstrated by invasion assays of HeLa cells. Higher concentrations (greater than or equal to 0.002%) were necessary to result in total bacterial death (114).

One study demonstrated that more plasmid DNA remained functional in water after peracetic acid (PROXITANE WW-12, Sigma-Aldrich) disinfection than after chlorination. The authors postulated that these functional genetic elements could be acquired by other microorganisms via horizontal gene transfer and pose potential public health and environmental risks (115).

Mechanism of resistance

There are no reports on specific mechanisms involved in resistance to peracetic acid. This is probably due to a lack of reports of resistance to the biocide. It has been shown that different ascospore-forming molds have different resistance patterns to peracetic acid (116). Although the resistant mechanism is unknown, the most resistant species, *Chaetomium globosum,* has thicker and more electron-dense walls compared to other mold species (117). This is unlikely to be an acquired trait, but the thicker cell wall may impede the action of peracetic acid (118).

Conclusion

Microorganisms in biofilms are much more resistant to decontamination by peracetic acid. Reports on the emergence of higher resistance towards peracetic acid following exposure to the biocide are extremely rare. *S.* Typhimurium LT2 cells were shown to resist disinfection by the biocide by remaining in a viable but nonculturable state. **No clinically relevant resistance has been identified yet.** The mechanisms of resistance are mostly unexplored.

Peracetic acid is an efficient biocide with a non-specific mode of action that readily decompose into non-toxic product in the environment. **All these features make peroxygen compounds such as peracetic acid very attractive disinfectants.**

Chlorhexidine

Introduction

Chlorhexidine is a cationic biguanide, mainly used in the form of its salts, namely chlorhexidine digluconate (CAS number 18472-51-0) or chlorhexidine diacetate (CAS number 56-95-1). Only chlorhexidine gluconate is authorized and under review for use as a PT1, PT2 and PT3 product in the EU. Chlorhexidine is used in washing and cleaning products, disinfectants, perfumes and fragrances, cosmetics and personal care products, polishes, waxes and pharmaceuticals. It can be found in paper-based products (such as tissues, feminine hygiene products, diapers, books, magazines, wallpaper). Professional workers in the healthcare field also use it as a hand scrub, a disinfectant for surgical sites, a disinfectant for mucous membranes and wounds, a surface disinfectant, and a disinfectant for instruments. It can also be used for the disinfection of burns and as a non-volatile active ingredient in alcohol-based hand wipes (119).

The first targets of chlorhexidine are the cytoplasmic membrane and membrane-bound enzymes, while secondary effects (at higher concentrations) are cytoplasmic leakage and, ultimately, the coagulation and precipitation of intracellular constituents such as proteins and nucleic acids (120). As the cytoplasmic membrane is the main action site of chlorhexidine, the outer membrane in Gram-negative bacteria may act as a permeability barrier for chlorhexidine and limit its antibacterial efficacy. The cationic chlorhexidine molecules may get stuck in the outer membrane due to interactions with the negatively charged lipopolysaccharide and thus may be prevented from reaching the cytoplasmic membrane (120).

Chlorhexidine is not sporicidal, although it prevents the development of spores. It has poor mycobactericidal activity and has low activity against most viruses, although lipid-enveloped viruses are more sensitive. It is generally active against other non-sporulating bacteria and yeasts (15). Nevertheless, extremely high MIC values (above 0.1%) have been described for many bacterial isolates, including isolates from *E. faecalis, K. pneumoniae, Proteus* spp.*, B. subtilis, P. aeruginosa, L. monocytogenes, E. faecium, S. aureus, Streptococcus* spp.*, S. marcescens, Acinetobacter* spp.*, Citrobacter* spp. and *Enterobacter* spp. The maximum epidemiological cutoff was proposed at 0.0064%. It is thus safe to say that amongst these species (and possibly others), clinically resistant isolates have already been discovered (119). Concerning bactericidal activity, 4% chlorhexidine has sufficient bactericidal activity (above 5-$\log_{10}$ reduction) against almost all non-sporulating bacterial species within 3-5 min except *Enterococcus* spp. Two percentchlorhexidine is still bactericidal but ineffective against some isolates of *E. faecium*, MRSA and *S. epidermidis*. At lower concentrations, bactericidal activity is variable (119).

Although chlorhexidine is popular in consumer products, there is mounting evidence that microorganisms can become resistant to this biocide, and there may be hints of cross-resistance to clinically important antibiotics as well. Here we describe studies which report such cases of increased resistance or cross-resistance to chlorhexidine, as well as the associated resistance mechanism. If mentioned in the study, we will report the form of chlorhexidine that was used in the experiment (digluconate or diacetate), but bear in mind that these salts are dissociated in water and the effect of the anion on resistance is likely to be extremely minor compared to the chlorhexidine cation.

Increased resistance in bacterial biofilms

There is a wealth of evidence that chlorhexidine is less effective at killing microorganisms in biofilms than in planktonic cultures. The MIC against chlorhexidine (oral rinse solution, Drogsan, Turkey) of ten bacterial species, including *S. mutans*, *Lactobacillus acidophilus*, *S. aureus* and *E. faecalis*, was between 2- and 128-fold higher in single species biofilms that for planktonic cells (121). *S. epidermidis* and *Staphylococcus capitis* were reported to be at least 64-fold more resistant to chlorhexidine (Sigma-Aldrich) than their planktonic counterparts (122). In one study, a high number of *S. mutans* cells (10^{10}) were completely eradicated by exposition to 0.01% of chlorhexidine, while biofilms of the same species required 0.0275% (123). The biofilms of clinical and environmental *A. baumannii* isolates were resistant to chlorhexidine (chlorhexidine digluconate, Sigma-Aldrich) concentrations that completely eradicated planktonic cells (124). In *B. cenocepacia*, low (0.0005%) and high (0.05%) concentrations of chlorhexidine (chlorhexidine gluconate solution, ABC Chemicals, Woutersbrakel, Belgium) had a similar effect on planktonic and sessile (biofilm) cells, but at intermediate concentrations (0.015%) the antimicrobial activity was more pronounced in planktonic cultures (about 75% of cells killed in planktonic cultures, only about 45% for sessile cells) (125). Following treatment with a commercial product containing 4% chlorhexidine (chlorhexidine gluconate, Medichem International), up to 11% of cells in MRSA biofilms survived, and up to 80% of cells in *P. aeruginosa* biofilms survived (126).

Eradicating multi-species biofilms with *S. aureus, E. faecalis, K. pneumoniae* and *P. aeruginosa* required between 4- and 16-fold the dose required of chlorhexidine digluconate (Sigma-Aldrich) for complete killing of the planktonic cells on their own. *K. pneumoniae* and *P. aeruginosa* survived within multi-species biofilms at 4% chlorhexidine, whereas *S. aureus* was reduced to below the level of detection at 1%. Interestingly, chlorhexidine-containing medical dressings completely eliminated *S. aureus*, but had a minimal effect (<3-$\log_{10}$ reduction) against the other species tested (127).

The age of the biofilm also has an impact on killing by chlorhexidine. Cultures of *Actinobacillus actinomycetemcomitans* were more resistant to 0.2% chlorhexidine (Sigma) when grown as a 3-day old biofilm than in a one-day old or planktonic cultures (128). In a multispecies biofilm grown from plaque bacteria for time periods ranging from 2 days to several months, it was shown that bacteria in mature biofilms (more than 2-week-old) and nutrient-limited biofilms are more resistant to killing by chlorhexidine than in young biofilms (less than 2-week-old) (129, 130).

This lower efficacy can be explained by different mechanisms. Some studies focus on physical mechanisms. Chlorhexidine from a commercial mouthwash product (PeridexTM, 3M ESPE, USA) at a 0.12% concentration was shown to penetrate a single-species biofilm of *S. mutans* rather slowly, at a velocity of just 6 µm/min. Unsurprisingly, this product was unable to eliminate the biofilms in the study (131). Treatment with chlorhexidine (0.03%) lead to a 1.8-$\log_{10}$ reduction of CFU in biofilms of nontypeable *Haemophilus influenzae* (while 99% of planktonic cells were killed with the same treatment). In contrast, biofilms that were scraped from the surface and dispersed by vortex agitation exhibited a 5 to 6-$\log_{10}$ unit decrease in CFU (132). These findings demonstrated that the inherent protective nature of the biofilm matrix and its ability to retard penetration of agents into the biofilm is a major element of biofilm resistance to chlorhexidine.

Increased resistance in fungal biofilms

Most studies focused on the *Candida* genus and found increased MIC for cells grown in biofilms compared to planktonic cells in *C. albicans, C. parapsilosis* and *C. auris* (79, 133-136).

In one study, 0.05% chlorhexidine was able to completely kill pure yeast suspensions of three clinical isolates of *C. albicans, Cryptococcus neoformans* and *Rhodotorula rubra*, and two environmental isolates of *C. albicans* and *Cryptococcus uniguttulatus*. However, a ten-fold increase in the concentration of chlorhexidine (0.5%) was unable to completely kill off the yeasts when they were mixed together in different combinations, even though it exhibited a substantial fungicidal activity. When yeasts were grown as biofilms, 0.5% chlorhexidine was unable to fully eliminate either the isolates alone or the mixed biofilms. Of note, chlorhexidine was the only agent tested that could reduce fungal load by more than 4-$\log_{10}$ yeast cell per mL (compared to betadine, sodium hypochlorite, 70% alcohol, 0.5% ecodiol) (137).

As is the case with bacteria, early biofilm were also more sensitive to chlorhexidine than mature biofilms (79).

There was little information on the possible mechanism of resistance of *Candida* species in biofilm, but one study found that *C. albicans* exhibited a strikingly biphasic killing pattern in response to chlorhexidine, indicating that a subpopulation of highly tolerant cells, termed persisters, existed. The surviving *C. albicans* persisters were detected only in biofilms and not in exponentially growing or stationary-phase planktonic populations, suggesting that these persisters are indeed not mutants but phenotypic variants of the wild type (138).

Emergence of resistance

Exposure of microbes to sub-inhibitory concentrations of chlorhexidine has been extensively covered in the literature. A comprehensive review of these data was published in 2018 in a book chapter (119) and in 2019 in a review article (139). This section sums up the data that were reviewed and provides more in-depth explanation on the cases where resistance is highest and when pre-exposure to chlorhexidine is associated with cross-resistance to other antimicrobials. New developments since 2018 and the mechanisms associated with resistance are also discussed. For a comprehensive table reporting all microorganisms that develop resistance, the associated increase in MIC value and potential cross-resistance, please refer to the book chapter (119).

There were reports of isolates or strains from 12 species of Gram-positive and 12 species of Gram-negative bacteria that displayed a weak adaptive response to exposure to sub-inhibitory concentrations of chlorhexidine (less than 4-fold increase in MIC). A strong response (more than 4-fold increase in MIC) was found in 2 isolates or strains of Gram-positive bacteria, and in 17 Gram-negative bacteria. The strongest MIC increase in Gram-positives was found in *S. aureus* (16-fold, with an MIC of 0.002%) and *E. faecalis* (6.7-fold, with an MIC of 0.0024%). Gram-negative bacteria, however, harbored the most striking changes: *E. coli* (500-fold), *Salmonella* spp. (200-fold), *S. marcescens* (128-fold) and *P. aeruginosa* (32-fold). They also had the highest MIC values after adaptation: *S. marcescens* (0.2%), *P. aeruginosa* (0.1%), *Salmonella* spp. (> 0.1%), *B. cepacia complex* (0.07%), *K. pneumoniae* (> 0.05%) and *E. coli* (0.05%) (139).

In one study, strains were repeatedly passaged in media containing increasing concentrations of chlorhexidine (Sigma), and the MIC was measured after each passage. The MIC of the parent strain of both *S. enterica* serovar Virchow and *E. coli* O157 began at 0.0004% and increased to 0.0128% and 0.0512% respectively after only 6 passages. In addition to becoming resistant to chlorhexidine, *S. enterica* serovar Virchow also displayed increased resistance to **tetracycline** and **triclosan**, and *E. coli* O157 to **triclosan** only (as measured by a reduction in the diameter of inhibition in disk diffusion assays) (140). A very low dose of chlorhexidine (Sigma) (0.0000024%, 200-fold lower than the MIC calculated in the study), moderately increased (about 2-fold) conjugal transfer of sulfonamide resistance from a complex sewage effluent community to an *E. coli* recipient (141).

A 5 min exposure to low concentrations of chlorhexidine (Sigma) (0.00001-0.0004%) led to about a 25-fold increase in the MIC of two *S. enterica* serovar Typhimurium strains. This increase in chlorhexidine resistance was coupled with a cross-resistance to **benzalkonium chloride** (about 60-fold compared to the WT). However, these resistances were not stable in time, as they were lost after 1 subculture in the absence of chlorhexidine. In the presence of a low chlorhexidine concentration, resistance levels also returned to normal after 10 subcultures. Furthermore, even though the increase in MIC is impressive, it remains below the levels that are likely to be obtained during use of consumer products. Indeed, none of the strains tested developed resistance when in contact with consumer products containing chlorhexidine (mouthwash and eye makeup remover) (142).

Salmonella isolates exhibiting a high-level resistance to chlorhexidine after adaptation (>0.1% in some case) were successfully obtained. The selected phenotype was stable, being maintained after several rounds of culture in the absence of the selective agent. In addition to their chlorhexidine resistance, one of the selected isolates was more resistant than their parent strain to **tetracycline** (16-fold), **chloramphenicol** (2-fold) and **nalidixic acid** (4-fold) (89).

One study compared modern strains of *K. pneumoniae* to strains that were isolated before the widespread use of chlorhexidine digluconate. They found that the modern strains had up to 4-fold higher resistance to chlorhexidine than the pre-chlorhexidine isolated strains. Furthermore, when the modern *K. pneumoniae* isolates were cultured in the presence of sublethal levels of chlorhexidine, five out of seven showed a stepwise increase in chlorhexidine MIC during the course of the serial selection, with final MICs being between 0.0128 and 0.0512%. Chlorhexidine-adapted strains showed decreased susceptibility to biocide formulations containing chlorhexidine. There was at most a 128-fold reduction of susceptibility for a commercial hand disinfectant containing 1-3% chlorhexidine, leading to resistance of some strains to more than 50% of the working concentration after 5 min of exposure. This is concerning, considering that the contact time recommended by the manufacturer is between 30 sec and 1 min. The authors conclude that not all commercially available formulations reach the minimum required concentration to achieve a satisfactory level of bacterial kill (143). Adaptation of *K. pneumoniae* to chlorhexidine was also shown to lead to **colistin** resistance, with an MIC increase from between 0.0002 to 0.0004% to more than 0.0064% (115, 144).

S. marcescens demonstrated an ability to grow in certain chlorhexidine-based disinfecting solutions (up to 0.006% chlorhexidine) recommended for rigid gas-permeable contact lenses. Cells that were inoculated into disinfecting solution went into a nonrecoverable phase within 24h, but after 4 days cells that had the ability to grow in the disinfectant emerged, even though the solution was still bactericidal to *P. aeruginosa*. For chlorhexidine-adapted cells, the MIC was 8-fold higher than for non-adapted cells. These chlorhexidine-adapted strains persisted or

grew in several other contact lens solutions with different antimicrobial agents, including **benzalkonium chloride** (0.004%) (145).

For *Burkholderia lata* strain 383, a high initial MIC of 0.07% was found to be unchanged by serial passage in sub-inhibitory concentrations of chlorhexidine gluconate (Sigma). The adapted strain was however less sensitive to **ciprofloxacin**, **tobramycin**, **ceftazidime**, **imipenem** and **meropenem**, as measured by a smaller diameter of inhibition in a disk diffusion susceptibility assay. While the data varied greatly between replicates, the increased susceptibility was not lost after several passages in the presence of low concentrations of chlorhexidine (146).

Following culture in sub-inhibitory concentrations of chlorhexidine (Sigma) for 14 days continuously, 6 isolates of *S. aureus* increased their MICs between 4- to 8-fold. Regardless of their ability to adapt to chlorhexidine, all isolates tested had a cross resistance to at least one antibiotic. Between the different isolates tested, there were increases in the MIC against **ciprofloxacin**, **tetracycline**, **gentamicin**, **amikacin**, **cefepime** and **meropenem**. All isolates became cross-resistant or showed increased resistance to tetracycline. Remarkably, a >512-fold increase in MIC to **amikacin**, **tetracycline** and **gentamycin** was found in one isolate (147). After exposure to a sub-inhibitory concentration of chlorhexidine, vancomycin-sensitive MRSA developed increased resistance to both chlorhexidine and **vancomycin** (an 8-fold increase in MIC) in the course of 50 days (148).

Reduced chlorhexidine susceptibility emerged (4-fold change in MIC) in vancomycin-resistant *E. faecium* strains after serial exposure to increasing concentrations of a chlorhexidine gluconate product (Hibiclens) for a period of 21 days. Subpopulation resistant to 0.001% **daptomycin** also emerged amongst the chlorhexidine-adapted strains (149).

Strains of *B. cepacia*, *E. faecalis*, *K. pneumoniae*, *S. marcescens*, *Staphylococcus lugdunensis*, and *Stenotrophomonas maltophilia* that were adapted in the presence of chlorhexidine (Sigma) (for 10 passages, they had at least a 4-fold increase in MIC). After growth in a microbicide-free environment, this adaptation was stable for *K. pneumoniae*, *S. maltophilia*, and *S. lugdunensis*. The maximum MIC was attained by *S. marcescens* at 0.012%, which is higher than the concentration of chlorhexidine in some commercial products. After stepwise exposure, *K. pneumoniae* and *S. marcescens* had about 2-fold increase in biofilm production ability, while *B. cepacia* displayed about a 2-fold decrease (150).

Bacteria that had been isolated from two high-risk environments, the skin and a domestic drain biofilm, were repeatedly exposed to chlorhexidine (Sigma) in a stepwise manner. Strains or isolates of *Aranicola proteolyticus, Pseudomonas sp., Ralstonia sp., S. maltophilia, Corynebacterium pseudogenitalum, Corynebacterium renale, Staphylococcus cohnii* and *S. lugdenensis* all had an increase in either their MIC or MBC values that ranged from 2-fold to about 16-fold. The maximum MIC value (0.017%), higher than the concentration of chlorhexidine in some commercial products, was obtained after chlorhexidine-adaptation of *Ralstonia* sp. (151).

Amongst 76 strains that were isolated from organic food, gradual exposure to chlorhexidine (Sigma) increased resistance (between 2- and 50-fold) to this biocide in 67 (88.2%) of them. These strains belonged to genera *Bacillus* (10 strains), *Enterococcus* (20 strains), *Staphylococcus* (8 strains), *Chryseobacterium* (2 strains), *Enterobacter* (10 strains), *Pantoea* (10 strains), *Salmonella* (3 strains), *Klebsiella* (3 strains) and Enterobacteriaceae (1 strain). Amongst those strains, five isolates (*B. cereus*, *Enterococcus casseliflavus*, *E. faecium*, *E. cloacae* and *Enterobacter sp*.) conserved their chlorhexidine resistance after 20 subcultures

in biocide-free broth. Most of the chlorhexidine-adapted strains showed higher resistance to **benzalkonium chloride** (95.5%) and **hexachlorophene** (94.0%) compared to the parent strains. Approximately 90% of adapted strains had higher resistance to **triclosan** or **didecyldimethylammonium bromide** and 80% were more resistant to **hexadecylpyridinium chloride** or **cetrimide**. Furthermore, most of the adapted strains (92%) had an increased resistance to at least one antibiotic. Resistance to **imipenem** was most frequently found in the adapted strains compared to wildtype, although high percentages of strains with increased resistance to **ceftazidime, sulfamethoxazol, tetracycline,** or **cefotaxime** were also detected (152).

Contamination of disinfection solutions

In a study, the surface of dispensers of hand soap with 2% chlorhexidine was contaminated with pan-resistant *Acinetobacter* and *Klebsiella* (these germs are resistant to all available antibiotics), multidrug-resistant *Pseudomonas*, and methicillin-resistant *S. aureus* (MRSA). The Gram-negative isolates could multiply in the presence of 1% chlorhexidine, but MRSA was inhibited *in vitro* by chlorhexidine at concentrations as low as 0.0019% (153). A 2% chlorhexidine hand-washing solution (Hibitane, AyerstLaboratories, Montréal), was contaminated by *S. marcescens,* probably surviving in a biofilm matrix. Viable *S. marcescens* cells were recovered from the solution over a period of 27 months. The MIC of this strain of *S. marcescens* was 0.1%, but it could survive in concentrations of up to 2% (20-fold the MIC) (154). A contamination of a chlorhexidine stock solution (unreported concentration) by *S. marcescens* was potentially at the origin of a small outbreak with a 26% lethality in a Mexican hospital (155). In 2014, twenty-one cases of bacteremia related to *S. marcescens* were identified in Madrid and contaminated commercial solutions of 0.05% and 2% chlorhexidine (BohmClorh healthy skin) were identified as the source of the outbreak (156).

In 2014, a post-operative wound infection caused by *S. marcescens* was diagnosed in 10 patients out of 54 patients that underwent open heart procedures during a 7-week period in a Spanish hospital. *S. marcescens* was isolated from the samples taken from all infected wounds. One patient died. The origin of the outbreak was determined to be the contamination by *S. marcescens* of the aqueous chlorhexidine solution (Bohm Laboratories) used to prepare the patients' skin (157).

A strain of *B. cepacia* that had colonized a 0.5% chlorhexidine gluconate solution was the source of an outbreak impacting 21 patients. The solution had been used as a skin antiseptic during blood drawing in the hospital (158). A polyclonal outbreak of *B. cepacia* complex was also caused by the contamination of multiple brands of commercial chlorhexidine solutions by *B. cepacia* complex and species of *Ralstonia,* impacting in total 53 patients at several Honk Kong hospitals between 2018 and 2019 (159).

Mechanism of resistance

Acquired genetically defined mechanisms conferring resistance toward chlorhexidine include multidrug efflux pumps and cell membrane changes. For instance, the *qac* (quaternary ammonium compound) gene family (*qacA*, *qacB*, *qacC/smr*) is well known in Gram-positive bacteria (mainly in staphylococci). These genes encode for efflux proteins that belong to the "Major Facilitator Superfamily" (MFS) or to the "Small Multidrug Resistance" (SMR) family (Smr) and have cationic biocides as substrates, including quaternary ammonium compounds and biocides (120). In Gram-negative bacteria, efflux proteins from the SMR family, from the "Resistance-Nodulation-Division" (RND) superfamily (like the Mex efflux systems in

P. aeruginosa), from the "Major Facilitator Superfamily", and from multiple less characterized families have been described (120), but resistance has also been linked to other stress responses. In this section the different occurrences where these mechanisms are linked to chlorhexidine resistance are outlined.

One study examined 94 clinical isolates of *S. aureus* and found that isolates with *qac* genes (encoding an efflux pump) had significantly higher MBCs for chlorhexidine and that the use of this biocide induced expression of *qac* genes when assayed with a luciferase reporter (160). A plasmid (PC02) that can be transferred between staphylococcal strains has been shown to harbor the *qacA* gene (161). The presence of both the *smr* and *qac* genes seems to have a synergistic impact on the MIC/MBC of *S. aureus* to chlorhexidine (162). *qac* resistance genes are common amongst *E. coli* isolated from retail meat, and are highly associated with resistance phenotypes (163, 164).

In *P. aeruginosa*, the RND-type MexCD-OprJ multidrug efflux pump is induced by sub-inhibitory concentrations of chlorhexidine, and its expression is dependent upon the AlgU stress response sigma factor. The development of chlorhexidine-resistant *P. aeruginosa* in biofilms also is dependent on the *mexCD-oprJ* genes (165). MexCD-OprJ appears to be the most important AlgU-regulated determinant of chlorhexidine resistance, although other AlgU-regulated factors seems to be necessary for full chlorhexidine resistance. Chlorhexidine resistant mutants selected following sub-inhibitory exposure of *P. aeruginosa* to chlorhexidine were recoverable only from a MexCD-OprJ$^+$ strain (166).

In *E. faecium*, a putative two-component system, composed of a putative sensor histidine kinase (ChtS) and a cognate DNA-binding response regulator (ChtR) was discovered and conferred resistance to chlorhexidine. The ChtRS two-component system was also necessary for resistance to bacitracin (167). Exposure of vancomycin-resistant *E. faecium* to chlorhexidine leads to a 10-fold upregulation of VanA-type vancomycin resistance (*vanHAX*) and genes associated with reduced daptomycin susceptibility (*liaXYZ*). However, in the presence of sub-inhibitory concentration of chlorhexidine, the strains were unexpectedly more sensitive to vancomycin, suggesting that chlorhexidine-induced gene expression changes lead to additional alterations in cell wall synthesis (168).

EfrEF is a heterodimeric ABC transporter that was shown to mediate the efflux of fluorescent substrates and confer resistance to multiple dyes and drugs, including fluoroquinolone (169). In one study that identified adaptive changes in chlorhexidine-adapted mutants which had a reduced daptomycin susceptibility, the role for EfrEF as a drug efflux system that reduces chlorhexidine susceptibility was confirmed. Its deletion increases susceptibility of *E. faecium* to chlorhexidine, and an amino acid substitution in EfrE is associated with decreased susceptibility to chlorhexidine. The presence of two ethoxylated fatty amine compounds was abolished in the *efrEF* deletion mutant relative to the wild type, but it remains to be determined whether and how these compounds are protective against chlorhexidine (149).

In *A. baumannii* the resistance/nodulation/division (RND) superfamily efflux systems AdeABC (*Acinetobacter* drug efflux) and AdeIJK, the major facilitator superfamily (MFS) AedF (*Acinetobacter* exporter of the DHA2 family) transporter, the small multidrug resistance (SMR) family exporter AdeS, as well as the *aceI* (*Acinetobacter* chlorhexidine efflux) gene have been shown to confer chlorhexidine resistance (170, 171).

Chlorhexidine resistance was linked in *K. pneumoniae* to the increased expression of *smvA*, an efflux pump of the major facilitator superfamily (MFS) and has been implicated in methyl

viologen resistance and the efflux of acriflavine and other quaternary ammonium compounds in *Salmomella enterica* serovar Typhimurium. In addition, mutation in *phoPQ* were also observed during chlorhexidine adaptation and could clearly be linked to colistin cross-resistance. However, the data from the study suggest that additional factors are important in mediating resistance to chlorhexidine, and they may operate independently of *phoPQ*, since overexpressing *phoPQ* in a parent strain led to colistin resistance, but not chlorhexidine resistance (144). KpnEF was characterized as an efflux pump that might help transport polysaccharides to the outer layer of bacterial cell to form the slimy layer and is possibly under additional regulation by other transcriptional factors involved in modulating capsule synthesis and biofilm formation in *K. pneumoniae*. Mutation in *kpnEF* resulted in increased susceptibility to cefepime, ceftriaxone, colistin, erythromycin, rifampin, tetracycline, and streptomycin and the Δ*kpnEF* mutant displayed enhanced sensitivity toward disinfectants such as benzalkonium chloride, chlorhexidine, and triclosan (172). However, it is not known whether the expression of this gene is induced in the presence of chlorhexidine.

A cation efflux pump gene, *cepA,* conferred chlorhexidine resistance when it was cloned into *K. pneumoniae* and *E. coli* (173). In 64 isolates of *K. pneumoniae*, 50 had reduced sensitivity to chlorhexidine, and this reduced sensitivity was associated with the presence of the *cepA* or *qac* efflux pump genes in 56 of these isolates (174). *cepA* was also found to be strongly upregulated in *K. pneumoniae* strains that had been adapted to chlorhexidine and were stably resistant to this antimicrobial (115). A *Klebsiella oxytoca* isolate from a diabetic foot ulcer with reduced sensitivity to chlorhexidine was found to harbour the *qacE* gene in a class I integron (175). The presence of *qacF* and *qacEΔ1* genes was significantly correlated with the chlorhexidine resistance of *E. coli* isolates for retail chicken (163).

A chlorhexidine- and multidrug-resistant strain of *Chryseobacterium indologenes* was shown to exhibit a 19-fold up-regulation of expression of the HlyD-like periplasmic adaptor protein of a tripartite efflux pump upon exposure to 0.0016% chlorhexidine suggesting that multidrug resistance may be mediated by this system (176).

In *B. lata,* exposure to chlorhexidine led to the overexpression of an ABC transporter (BCAS0081, 102-fold) and an RND efflux pump (BCAM2551, 2.5-fold) (146), indicating that efflux might also be a main resistance mechanism for this species.

Another study focused on the response and resistance of *B. cenocepacia* biofilms to chlorhexidine compared to planktonic cells. Treatment with chlorhexidine increases the expression of several genes encoding membrane-related and regulatory proteins, as well as several genes coding for drug resistance determinants (including RND and MFS efflux systems). The chlorhexidine resistance mechanisms described here are lifestyle-specific, as some efflux pumps were responsible for chlorhexidine efflux in planktonic cells while others were responsible for chlorhexidine efflux in sessile cells. The downregulation of a gene encoding an adhesin and the upregulation of many genes encoding chemotaxis and motility-related proteins may indicate that sessile cells try to escape from the biofilm upon chlorhexidine exposure (125).

Conclusion

Chlorhexidine is a popular biocide. Adaptation of bacterial strains to this biocide has been shown countless times, especially for Gram-negative bacteria which can reach clinically relevant levels of resistance. Bacteria (as well as yeasts) in biofilms are especially resistant.

The microorganism to monitor because of their proven high development of resistance, high level of resistance, or potential to develop cross-resistance are *E. faecalis, K. pneumoniae, Proteus* spp.*, B. subtilis, P. aeruginosa, L. monocytogenes, E. faecium, S. aureus, Streptococcus* spp.*, S. marcescens, Acinetobacter* spp.*, Citrobacter* spp., *Enterobacter* spp., *Salmonella* spp., and *B. cepacia.* More information on the emergence of resistance for these species and others can be found in the main body of the report.

There are many reports of different bacterial isolates developing cross-resistance to other biocidal products or antibiotics after exposure to chlorhexidine. Table 3 is a summary of the data that was reported in this literature review. More information on cross-resistance following the use of chlorhexidine can be found in the main body of this report.

Table 3 - reports of cross-resistance following exposure to chlorhexidine

Organism	Antimicrobial
Salmonella	Tetracycline, triclosan, benzalkonium chloride, chloramphenicol, nalidixic acid.
Escherichia	Triclosan
Klebsiella	Colistin
Burkholderia	Ciprofloxacin, tobramycin, ceftazidime, imipenem, meropenem
Staphylococcus	Ciprofloxacin, tetracycline, gentamycin, amikacin, cefepime, meropenem, vancomycin
Enterococcus	Daptomycin
Organic food isolates	Different levels of increased resistance to benzalkonium chloride, hexachlorophene, triclosan, didecyldimethylammonium bromide, hexadecylpyridinium chloride, cetrimide, imipenem, ceftazidime, sulfamethoxazol, tetracycline, cefotaxime

The mechanism of resistance to chlorhexidine is not completely elucidated and is different for different bacterial species but seems to mainly involve modification of the bacterial membrane and the expression of efflux pumps such as the MexCD-OprJ, KpnEF, EfrEF and Qac efflux pumps. Other pleiotropic effect through more global regulators may also be involved. These mechanisms are mostly nonspecific and the high prevalence of efflux pump determinants in chlorhexidine-resistant strains is indicative of the high number of cross-resistance to other antimicrobials (including antibiotics) that are reported in association with chlorhexidine resistance.

That being said, chlorhexidine has been used for more than 50 years, and in real-world applications, while the use of chlorhexidine is linked with the emergence of resistance in bacteria, it does not seem to be a major source of outbreaks, although some occurrences have been described. Nevertheless, the use of chlorhexidine should be restrained to the applications where its greater efficacy has been proven compared to other biocides that are less associated with bacterial resistance. When used, good practice should be followed such that microorganisms are not needlessly exposed to sublethal concentrations of the biocide, a situation which breeds development of resistance.

Quaternary ammonium compounds

Surface-active agents (also called surfactants) have a hydrocarbon, water-repellent (hydrophobic) group region and a water-attracting (hydrophilic or polar) region. Depending on the charge of the hydrophilic group, surfactants may be classified into cationic, anionic, nonionic, and ampholytic compounds. Of these, the cationic agents, as exemplified by quaternary ammonium compounds (QACs, also known as cationic detergents), are the most useful antiseptics and disinfectants (15). These QACs are synthesized on an industrial scale, but are also commonly found in nature, where microorganisms are thought to synthesize these compounds to better adapt to changing environmental conditions (177). Benzalkonium chloride is a widely used QAC and as such will be used as an example in this report. Benzalkonium chloride is a mixture of alkyl benzyl dimethyl ammonium chlorides, in which the alkyl group has various even-numbered alkyl chain lengths. Benzalkonium chloride mixtures comprise of 24 compounds that are structurally similar QACs characterized by having a positively charged nitrogen covalently bonded to three alkyl group substituents and a benzyl substituent (178). Within the European Union, three mixtures have been notified as biocidal agents: a C12-C18 mixture (CAS number 68391-01-5), a C12-C16 mixture (CAS number 68424-85-1) and a C12-C14 mixture (CAS number 85409-22-9) (179).

QACs are used for a variety of clinical purposes such as hand scrubbing, preoperative disinfection of unbroken skin, application to mucous membranes, and disinfection of noncritical surfaces. Outside of hospitals, QACs are also used as surface disinfectants in household and foodservice settings, comprising the active ingredient of many commercially-available cleaning sprays and wipes (15, 178, 180). QACs are extensively used in SARS-CoV-2-related sanitization in clinical and household settings, which highlights the need to study the potential for the emergence of biocide and antibiotic resistances related to increased use of these compounds (180).

QACs are membrane-active agents effective against non-sporulating bacteria, with a target site predominantly at the cytoplasmic (inner) membrane in bacteria or the plasma membrane in yeasts (15). The cationic group facilitates cation adsorption on the cell surface (usually negatively charged) and the penetration of hydrophobic chains into the membrane, which causes its destruction and the leakage of intracellular substances such as potassium ions or DNA. They can affect the fluidity of the phospholipid bilayer, interfere with specific hydrophobic and electrostatic interactions between membrane proteins and lipids, and alter the asymmetry of lipid arrangement. In low concentrations, QACs can change the properties of proteins, while in high concentrations they lead to solubilization of proteins and lipids, which causes membrane destruction (177). QACs are sporostatic: they inhibit the outgrowth of spores but not the germination processes. QACs are not mycobactericidal but have a mycobacteriostatic action, although the actual effects on mycobacteria have been poorly studied. QACs have an effect on lipid enveloped viruses (including SARS-CoV-2, human immunodeficiency virus and HBV), but not on non-enveloped viruses (15, 181).

Concerning bacteria, the highest MIC values for benzalkonium chloride were described with *A. hydrophila* (up to 3.1%), *B. cereus* and *E. meningoseptica* (up to 0.78%) *P. aeruginosa* (up to 0.5%), *L. monocytogenes, E. cloacae* (up to 0.05%), *A. xylosoxidans* and *B. cepacia* (up to 0.05%) and *Proteus mirabilis* (up to 0.04%). These values are largely higher than the proposed epidemiological cutoff (below 0.0064% for most species) (178), indicating that some level of resistance is already widespread. Of note, with benzalkonium chloride the result of MIC testing

depends to some extent on the media composition and plate material showing the need to standardize biocide susceptibility testing (178).

Here, we analyze the relevant literature regarding the emergence of resistance to QACs in microorganisms, including the bacterial species concerned, the potential for cross-resistance and the mechanisms of resistance. We focused the research on benzalkonium chloride mixtures as it is one of the most widely used QAC and abundant literature is available, although data on other QACs have been included as well. The vast majority of studies do not mention the CAS number of the chemicals they use. We will try to provide as much information as possible, but indeed the exact identity of the substance under investigation is not always crystal clear. We still included data on benzalkonium chloride in this report as we (and others before us) consider it highly unlikely that a specific mixture of alkyl benzyl dimethyl ammonium chlorides would yield results that are not typical for the entire group of mixtures (178), especially regarding the emergence of resistance against such compounds.

Increased resistance to disinfection in biofilms

As is the case for most biocides, it has been described multiple times that bacteria in biofilms are inherently more resistant to benzalkonium chloride than bacteria that remain in the planktonic state. Additionally, multi-species biofilms and mature biofilms seem to display more resistance. In this section we summarize the different occurrences of this phenomenon.

In different strains of uropathogenic *E. coli,* the concentration of benzalkonium chloride (Sigma) required to eradicate biofilm was often more than 10-fold higher than the concentration required to kill planktonic cells (182). The proportion of *S. enterica* benzalkonium chloride-adapted biofilm cells able to survive a lethal benzalkonium chloride (Sigma) treatment (0.003%) was significantly higher (4.6-fold) than that of benzalkonium chloride-adapted planktonic cells. There was no statistical difference in survival when comparing the survival of non-adapted biofilm and planktonic cells (183).

Planktonic cells of isolates of *P. aeruginosa* from two dairies were about 3-fold more sensitive to benzalkonium chloride than biofilm of these strains when these were in contact with the biocide for 5 min. The longer the contact time, the lower the concentration needed to eradicate biofilms. For a contact time of 60 min, biofilms of the different isolates were killed by 0.04-0.07% benzalkonium chloride, compared to 0.025-0.035% for planktonic cells (184). In another study, planktonic cells were 100-fold more sensitive than cells in biofilms after 5 min of contact. Benzalkonium chloride (C14, Fluka, France) displayed slow penetration inside the cell and cells isolated from a biofilm did not display any significant differences in terms of their resistance to benzalkonium chloride, indicating that the exopolymeric matrix plays a major role in resistance of biofilms to this biocide (185). *P. aeruginosa* embedded in artificial biofilm were 1900-fold more resistant to benzalkonium chloride than planktonic cells. Increasing the concentration of the biocide (ADBAC, Barquat MB80-80%, Lonza, which contains predominantly C12 – C14 alkyl groups) increased bacterial killing more in the biofilm than in a suspension culture (47).

Planktonic cells of *L. monocytogenes* were also more sensitive to benzalkonium chloride than sessile cells (101, 186, 187), even in a strain that was previously adapted to benzalkonium chloride. The older the biofilm, the more resistant it is to benzalkonium chloride (Fragon Iberica S.A.U. Terrassa) (101). Increased resistance of *L. monocytogenes* in biofilm to benzalkonium chloride (Merck) was linked to the presence of *hrcA* and *dnaK* which encode the transcriptional regulator of the class I heat-shock response and a class I heat-shock response chaperone protein, respectively (103). *L. monocytogenes* strain C719 is at least 1000-fold more resistant to

benzalkonium chloride in biofilms than in planktonic form. Cells present in biofilms were shown to recover and grow after treatment, providing a source of recontamination (188). In one study, when different *L. monocytogenes* strains were challenged with a QAC (Supermix Sanitiser, Applied, Victoria, Australia) for 60s, increased survival was only observed in mature biofilms (more than 48h incubation), irrespective of whether the strain produced high or low amounts of biofilm, suggesting that the nature (early or mature biofilm) of the biofilm is more important than its quantity when it comes to resistance to QACs (189).

Mixed species biofilm have been shown to be more resistant to benzalkonium chloride as described in a study on biofilms formed of *L. monocytogenes* and *L. plantarum* where mixed-species biofilms were more resistant to benzalkonium chloride (Merck) than single-species biofilms (108). A *S. liquefaciens* and *S. putrefaciens* dual-species biofilms showed also stronger resistance to benzalkonium chloride than the mono-species biofilms. Moreover, a structural observation of the biofilms indicated that the extra-cellular polymeric substance (EPS) may play an important role in the protection of dual-species biofilm. Benzalkonium chloride (0.01%) was as effective as ethanol (75%) in killing bacteria, but ethanol was more effective in removing the biofilm (190).

Different strains of staphylococci (*S. capitis*, *S. cohnii*, *S. epidermidis*, *S. lentus*, and *S. saprophyticus*) isolated from food processing environment were more susceptible to benzalkonium chloride (Sigma–Aldrich) in suspension than in biofilm. Strains that formed protein-dependent biofilms (degraded by proteinase) were more susceptible to benzalkonium chloride than strains producing a biofilm of polysaccharide matrix (degraded by the glycoside hydrolase Dispersin B). Interestingly, there was no difference in susceptibility between strains containing *qac* genes (that encode an efflux pump known to confer resistance to benzalkonium chloride) and the other strains (191).

Emergence of resistance

Exposure of microbes to sub-inhibitory concentrations of quaternary ammonium compounds has been extensively covered in the literature. A comprehensive review of these data (focused on benzalkonium chloride) was published in 2018 in a book chapter (178) and in 2018 in a review article (179). This section sums up the data that was reviewed and provides extra explanation on the cases where resistance is highest and when pre-exposure to quaternary ammonium compounds is associated with cross-resistance to other antimicrobials. New developments since 2018 and the mechanisms associated with resistance are also discussed. For a comprehensive table reporting all microorganisms that develop resistance and the associated increase in MIC value and potential cross-resistance, please refer to the book chapter (178).

There was a strong change in MIC after adaptation in strains or isolates of *Pantoea* spp.*, Enterobacter* spp.*, S. saprophyticus* and *E. coli*. The highest MIC values after adaptation were 0.3% (*S.* Typhimurium), 0.25% (*P. aeruginosa*), 0.15% (*Enterobacter* spp.) and 0.1% (*E. coli and S. saprophyticus*). There were reports of cross-resistance to other biocides and antibiotics in some isolates (178).

S. enterica serovar Typhimurium strains SL1344 and 14028S had between 20- and 100-fold increase in MIC for benzalkonium chloride (Sigma-Aldrich) after sublethal exposure to the biocide. However these strains did not develop any increase of resistance when exposed to the biocide in a formulation (a shampoo) (142). *S. enterica* biofilms adapted to benzalkonium chloride (Sigma) over a 144h-period had 18.3-fold more survivors than among their non-adapted counterpart and could survive a normally lethal benzalkonium chloride challenge and

then regrow, while exposure of untreated control biofilms to the lethal benzalkonium chloride challenge resulted in biofilm erosion and cell death (183). Benzalkonium chloride-adapted (Sigma-Aldrich) mutant strains of *S.* Typhimurium (with a maximum of 16-fold increase in MIC compared to parent strain) also had between 2- and 8-fold increased resistance to **chloramphenicol**, **ciprofloxacin**, **nalidixic acid**, and **tetracycline** (192).

Strains of *S. enterica* and *E. coli* O157 had increased resistance to benzalkonium chloride (Sigma-Aldrich) after 6 passages in increasing concentration of the biocide and displayed increased resistance to other biocides/antibiotics. In particular, the *E. coli* O157 adapted strain was also more resistant to **amoxicillin**, **amoxicillin-clavulanic acid**, **chloramphenicol**, **imipenem**, **tetracycline** and **trimethoprim** (140).

E. coli adapted to benzalkonium chloride had an 8-fold MIC increase compared to the parental strain. This increased resistance to benzalkonium chloride was accompanied by cross-resistance to other antibiotics of at least 4-fold to **ciprofloxacin**, **ceftiofur**, **florfenicol**, and **cefotaxime** and a 16-fold increase in resistance to **chloramphenicol** (193). In different strains of uropathogenic *E. coli,* exposure to a sub-inhibitory concentration of benzalkonium chloride (Sigma-Aldrich) led to no increase in the MIC for benzalkonium chloride, but there was increased biofilm formation, and strains that were sensitive to ciprofloxacin became resistant (182).

The gradual exposure of *P. aeruginosa* to increasingly higher concentrations of benzalkonium chloride led to an increase in MIC from 0.0025% to 0.058% (a 20-fold increase). Interestingly, the benzalkonium chloride-adapted strain seemed to have slightly increased resistance to different antibiotics (**amikacin**, **ceftazidime**, **ciprofloxacin**, **gentamycin** and **imipenem**) compared to a non-adapted strain in a plate diffusion assay (194). A *P. aeruginosa* continuous culture enriched with benzalkonium chloride (Sigma-Aldrich) over the course of 792h (33d) yielded a strain that was 12-fold more resistant to the biocide (with an MIC over 0.035%). This variant also demonstrated a 256-fold higher resistance to ciprofloxacin, possibly due to a mutation in the *gyrA* gene (195).

Out of 16 *P. aeruginosa* strains (including 14 clinical isolates), 15 displayed increased resistance to benzalkonium chloride (Sigma-Aldrich) after adaptation through serial passage, with a maximum of 10-fold increase and a MIC of 0.05% (which was the maximum concentration tested). Amongst these adapted strains, two showed a stable increase in resistance. Co-resistance to other quaternary ammonium compounds was observed in both strains and **chloramphenicol** and **polymyxin B** resistance (2-fold and 10-fold, respectively) was observed in one (196). Similarly, when 43 *P. aeruginosa* clinical isolates were subjected to increasing sub-lethal concentrations of benzalkonium chloride, the adapted isolates showed a moderate increase in antibiotic resistance (of max. 8-fold, to **ampicillin**, **cefotaxime**, **cefepime**, **amikacin**, **gentamycin**, **tetracycline**, **ciprofloxacin**, **lomefloxacin**, **trimethoprime** and **imipenem**). 66% of the isolates showed retardation of growth, 63% showed increased cell surface hydrophobicity and 23.5% exhibited enhanced biofilm formation (197) (Sigma-Aldrich).

Incubating lactic acid bacteria (strains of *Lactobacillus pentosus*) with low dose of benzalkonium chloride (Sigma-Aldrich) led to increase in the MIC of several antibiotics, including **ampicillin** (up to 100-fold), **chloramphenicol** (up to 500-fold) and **tetracycline** (up to 80-fold) (198).

Short-term exposure of *B. lata* to 0.005% benzalkonium chloride (Sigma) can lead to a decrease in the zone of inhibition for **ceftazidime**, **ciprofloxacin**, **meropenem** and **imipenem** (146).

Strains of *P. aeruginosa, S. aureus, E. coli, A. baumannii, P. putida* exhibited a >4-fold increase in MIC after exposure to benzalkonium chloride (Sigma-Aldrich), and this increase was stable in the absence of the biocide for *E. coli* and *P. aeruginosa* (199). Strains of *Aranicola proteolyticus,* and *Ralstonia* sp. had a MIC increase of more than 10-fold following exposure to increasing concentrations of two different quaternary ammonium compounds (Bardac 2250 and Barquat MB80, Lonza) (151).

In one study, 76 biocide-sensitive bacterial isolates from organic food were exposed to increasing concentrations of benzalkonium chloride (Sigma-Aldrich). Benzalkonium chloride resistance increased between 2- and 100-fold in 88.2% of strains. Gram-positive strains of *B. cereus* and *Staphylococcus*, and Gram-negative strains of *Enterobacter* and *Pantoea* showed MIC increases higher than 100-fold. Adaptive resistance was stable after 20 subcultures in biocide-free medium for 7 strains. Benzalkonium chloride-adapted strains often had reduced susceptibility to other biocides, including **chlorhexidine**, and showed increased resistance to antibiotics, including **ampicillin**, **sulfamethoxazole** and **cefotaxime** (200).

Exposure of biofilm cells of *Salmonella* Enteritidis NCTC 13349 to sublethal concentration of benzalkonium chloride (0.04%) (Sigma-Aldrich) led to an upregulation of virulence genes (*invA*, *avrA* and *csgD*) (85).

Strains of *L. monocytogenes* isolated from a poultry plant had about a 4-fold increase in MIC when repeatedly exposed to quaternary ammonium compounds (alkyl-benzyl-dimethyl ammonium chloride (Goldschmidt, Pandino, Italy) and n-alkydimethyl ethylbenzyl ammoniumchloride (Pointing Chemicals, Huddersfield, UK)). These adapted strains also displayed increased resistance (2-fold increase in MIC) towards **sodium hypochlorite** (active chlorine 10%) (Finnish Chemicals, Äetsä, Finland) (201).

50 *Pseudomonas* clinical isolates were exposed to the maximal sub-inhibitory concentration of a quaternary ammonium compound (didecyldimonium chloride, Virusolve+ concentrate 100%, actual concentration used was not communicated) overnight. After this incubation, the MIC against different antibiotics (**piperacillin-tazobactam**, **ceftazidime**, **gentamicin**, **amikacin**, **ciprofloxacin**, **meropenem** and **colistin**) was measured. For each of these antibiotics, there were at least a few isolates that displayed increased resistance. Furthermore, for **colistin**, **meropenem** and **ceftazidim**, there was at least one isolate that was above the threshold for clinical resistance for *Pseudomonas* according to the Clinical and Laboratory Standards Institute (2016) (202). Kim *et al.* (203) showed that exposure to benzalkonium chloride (mixture consisting of a 60:40 mixture of benzyldimethyldodecylammonium chloride and benzyldimethyltetrade-cylammonium chloride, C_{12}BDMA-Cl and C_{14}BDMA-Cl, respectively; Sigma-Aldrich) co-selects for benzalkonium chloride and antibiotic-resistant bacteria. After adaptation to benzalkonium chloride, *P. aeruginosa* developed higher resistance towards the biocide (up to 0.16%) that was accompanied with higher resistance to **polymyxin B** (2 to 8-fold increase in MIC, up to 0.00016%). The authors conclude that the MIC values against benzalkonium chloride obtained after adaptation are comparable to, or even higher than, those used in practice as a disinfectant and suggested that the accumulation of benzalkonium chloride in any nontarget environment (freshwater or sediment habitats or the waste stream of hospitals or food processing facilities) should be prevented to limit the spreading of antibiotic resistance determinants.

After exposure of *Cronobacter sakazakii* and *Yersinia enterocolitica* to increasing sub-inhibitory concentrations of benzalkonium chloride (Sigma-Aldrich), the strains exhibited resistance to **ciprofloxacin**, **cefotaxime** and **cefoxitin** (204).

Mechanisms of resistance

Bacterial resistance to QACs was first identified in the 1980s, but the precise mechanisms by which this resistance occurs remain largely understudied. Because the vast majority of QAC disinfectants function *via* membrane destabilization leading to cell lysis, Gram-negative bacteria have intrinsic resistance to QACs. In Gram-positive bacteria, acquired resistance is often the result of the presence of multidrug or QAC-specific efflux pumps. Common determinants of the resistance to QACs are plasmid-based *qac* genes (*qacA*, *qacB*, *qacC*, *qacE*, *qacEv1*, *qacG*, *qacH*, *qacJ*, *cepA*) and the *bcrABC* gene cassette encoding efflux pump systems (177, 180). Resistance of bacteria to QACs thus usually involves the expression of efflux pump and modification of the bacterial membrane, as well as the expression of various genes involved in stress response or antibiotic resistance. Some of these phenomena are described below.

Indeed, bacterial outer membrane proteins (OMPs) and lipopolysaccharide (LPS) may be involved in resistance to QACs. It has been shown that wild-type strains of *E. coli*, with no defect in OMP or LPS were resistant to QACs, while LPS-deficient strains had higher sensitivity (8). Repeated exposure of *E. coli* to benzalkonium chloride resulted in significant alterations in global gene expression. There was increased expression of genes associated with efflux and reduced expression of genes associated with outer membrane porins, motility, and chemotaxis. These changes resulted in minor decreases in biocide susceptibility, reductions in growth rate and biofilm formation, and loss of motility (10). *E. coli* K-12 adapted to benzalkonium chloride acquired several general resistance mechanisms including responses normally related to the multiple antibiotic resistance (Mar) regulon and protection against oxidative stress (193). *P. aeruginosa* ATCC 15442 (a strain recommended by ATCC for use in the test described in ASTM Standard Test Method E686-91) was able to adapt to increasing concentrations of a C14 QAC, benzyldimethyltetradecylammonium chloride. The C14-adapted cells showed variations in membrane fatty acid composition and a relationship was shown between the membrane fatty acids and the resistance developed by the strain against the bactericidal activity of C14 (205), indicating that the fatty acid composition of the membrane plays an important role in the bacterial resistance against QACs.

In biofilms of *S. enterica,* the proteins found to be up-regulated following benzalkonium chloride adaptation of a biofilm were involved in energy metabolism, amino acid and protein biosynthesis, nutrient binding, adaptation, and detoxification. A putative universal stress protein was also found to be up-regulated. Proteins involved in proteolysis, cell envelope formation, adaptation, heat shock response and broad regulatory functions were found to be down-regulated (183). A *S. enterica* serotype Hvittingfoss S41 that was adapted to benzalkonium chloride also displayed increased resistance (more than 4-fold) to multiple antibiotics, including ampicillin, piperacillin, tetracycline, ciprofloxacin, chloramphenicol, cefoxitin and nalidixic acid. Interestingly, the *in vitro*-selected benzalkonium chloride-resistant isolate and its susceptible parent were both inhibited at 6.25% of the working concentration of a commercial formulation containing benzalkonium chloride (89), suggesting that resistance to an active agent does not equal resistance to a biocidal formulation containing this active agent.

In two benzalkonium chloride-adapted *P. aeruginosa,* increased resistance was linked to alterations in outer membrane proteins, the uptake of benzalkonium chloride, cell surface charge and hydrophobicity, and fatty acid content of the cytoplasmic membrane. However, each of the two strains had different alterations in these characteristics, indicating that this adaptation is unique to each strain and does not result from a general mechanism (196). The resistance phenotype of an adapted strain of *P. aeruginosa* was mainly driven by an increased efflux activity. Overexpression of both MexAB-OprM and MexCD-OprJ was recorded and an amino acid substitution (Val-51→Ala) was observed in *nfxB*, the Mex efflux system regulator gene. Similarly, *mexR*, a repressor of the Mex system, was downregulated (195). In *P. aeruginosa* strains collected from clinical samples, veterinary samples, and wastewater, a subpopulation was resistant to benzalkonium chloride and showed cross-resistance to fluoroquinolones, cephalosporins, aminoglycosides, and multidrug resistance. Amongst this subpopulation, the epidemiological high-risk ST235 clone was the most abundant. The overexpression of the MexAB-OprM drug efflux pump resulting from amino acid mutations in regulators MexR, NalC, or NalD was the major contributing factor for cross-resistance that could be reversed by an efflux pump inhibitor (206). 60% of the benzalkonium chloride-adapted isolates of *P. aeruginosa* showed overexpression of *ndvB* biofilm gene, suggesting that these strains may use biofilm formation as a resistance strategy (197).

A novel genomic island (LGI1) was discovered in *L. monocytogenes* isolates responsible for the deadliest listeriosis outbreak in Canada in 2008. A putative efflux pump, *emrE* improved adaptation and growth of *L. monocytogenes* in the presence of quaternary ammonium compounds. The expression of *emrE* and several other genes on LGI1 is induced in the presence of benzalkonium chloride, and deletion of *emrE* results in reduced MICs and impaired growth and survival in the presence of quaternary ammonium compounds (207). The persistence of *L. monocytogenes* in a pig slaughterhouse was linked to the presence of the *bcrABC* cassette which is known to produce efflux pump-mediated benzalkonium chloride resistance (208). This plasmid-borne disinfectant resistance cassette can additionally be transferred between pathogenic and non-pathogenic strains of *Listeria,* suggesting that nonpathogenic *Listeria* spp. may behave as reservoirs for disinfectant resistance genes for other listeriae, including the pathogenic species *L. monocytogenes* (209). Indeed, out of 1,279 well-characterized *L. monocytogenes* isolates from various foods and food manufacturing environments, 531 (41.5%) isolates were found to harbor the *bcrABC* gene cassette (210). Martínez-Suárez *et al.* (211) note that although there are several well-characterized efflux pumps that confer resistance to QACs, it is usually a low-level resistance that does not generate resistance to QACs at the concentrations applied in real-world applications. However, dilution in the environment and biodegradation result in QAC concentration gradients. As a result, the microorganisms are frequently exposed to sub-inhibitory concentrations of QACs. Therefore, the low-level resistance to QACs in *L. monocytogenes* may contribute to its environmental adaptation and persistence. In fact, in certain cases, the relationship between low-level resistance and the environmental persistence of *L. monocytogenes* in different food production chains has been previously established. The resistant strains would have survival advantages in these environments over sensitive strains, such as the ability to form biofilms in the presence of increased biocide concentrations.

Other quaternary ammonium compounds

Other quaternary ammonium compounds can be used as disinfectants and are under review for use as PT1 and/or PT2 products in the EU, including Didecyldimethylammonium chloride (DDAC, CAS number 7173-51-5), DDAC (C8-C10, CAS number 68424-95-3), Alkyl (C12-

C14) dimethyl(ethylbenzyl)ammonium chloride (ADEBAC (C12-C14), CAS number 85409-23-0), Dimethyloctadecyl[3-(trimethoxysilyl)propyl]ammonium chloride (CAS number 27668-52-6), Polymeric quaternary ammonium chloride (PQ Polymer, CAS number 25988-97-0). Concerning the emergence of resistance against these biocides, very little information is available in the literature and more research is warranted for the biocides that have widespread use. A few research papers have documented the adaptation of bacterial strains to increasing concentrations of DDAC (PT1 and PT2). The information is summarized below. The CAS number of the chemical used was not mentioned in any of the papers, so the exact form of DDAC that was used is unknown.

Staphylococcus epidermidis strains that were adapted to increasing concentrations of DDAC had an increase in MIC values up to 180-fold, with a maximum MIC value of 0.0036%. Some of the DDAC-adapted strains displayed increased resistance to benzalkonium chloride and antibiotics such as gentamicin, erythromycin, ciprofloxacin, chloramphenicol and tetracycline, to a level above the clinical threshold for antibiotic resistance. The majority of adapted strains showed modifications to cell size and fatty acid composition and some of the adapted strains showed changes in biofilm formation and overexpression of efflux pumps (212). After adaptation to DDAC of 136 food-associated bacterial isolates, a 3-fold increase in the MIC values was observed in 48% of the *E. coli* and *L. monocytogenes* strains, and 3% of the *Salmonella* strains. Reduced susceptibility to benzalkonium chloride was also commonly found for all species except *Salmonella*. Cross-resistance to ampicillin, cefotaxime, ceftazidime, chloramphenicol and ciprofloxacin was also observed (213). Adaptation of a *P. fluorescens* strain to DDAC led to a 5-fold increase in MIC as well as increased cross-resistance to other disinfectants, including benzalkonium chloride (214).

Conclusions

Quaternary ammonium compounds are widely used. Adaptation of bacterial strains to higher concentrations of this biocide has been demonstrated many times, and some strains reach levels of resistance that are clinically relevant.

The microorganisms to monitor because of their proven high development of resistance, high level of resistance, or potential to develop cross-resistance include *A. hydrophila*, *B. cereus, E. meningoseptica*, *Pseudomonas* spp., *L. monocytogenes, E. cloacae*, *A. xylosoxidans*, *B. cepacia*, *P. mirabilis, Staphylococcus* spp*., E. coli, Salmonella* spp., *Enterobacter* spp., *Pantoea* spp., *L. pentosus*. More information on the emergence of resistance for these species and others can be found in the main body of the report.

There are many reports of different bacterial isolates developing cross-resistance to other biocidal products or antibiotics after exposure to chlorhexidine. Table 4 is a summary of the data that was reported in this literature review. More information on cross-resistance following the use of chlorhexidine can be found in the main body of this report.

Table 4 - reports of cross-resistance following exposure to QACs

Organism	Antimicrobial
Salmonella	Chloramphenicol, ciprofloxacin, nalidixic acid, tetracycline
Escherichia	Amoxicillin, amoxicillin-clavulanic acid, chloramphenicol, imipenem, tetracycline, trimethoprim, ciprofloxacin, ceftiofur, florfenicol, cefotaxime, chloramphenicol
Pseudomonas	Amikacin, ceftazidime, ciprofloxacin, gentamycin, imipenem, chloramphenicol, polymyxin B, piperacillin-tazobactam, amikacin, meropenem, colistin
Lactobacillus	Ampicillin, chloramphenicol, tetracycline
Burkholderia	Ceftazidime, ciprofloxacin, meropenem, imipenem
Organic food isolates	Chlorhexidine, ampicillin, sulfamethoxazole, cefotaxime
Listeria	Sodium hypochlorite
Cronobacter sakazakii, Yersinia enterocolitica	Ciprofloxacin, cefotaxime, cefoxitin
Staphylococcus	Gentamicin, erythromycin, ciprofloxacin, chloramphenicol, tetracycline

The mechanism of resistance seems to be centered around the expression of efflux pumps and modification of the bacterial membrane. These mechanisms are mostly nonspecific which is coherent with the numerous reports of cross-resistance to other biocides and antibiotics.

All things considered, while the use of quaternary ammonium compounds has been reported to be associated with the emergence of resistance in bacteria, it has not been linked to major outbreaks yet. **Nevertheless, the use of QACs such as benzalkonium chloride should be restrained to the applications where its greater efficacy has been proven compared to other biocides that are less associated with bacterial resistance**. When used, good practice should be followed such that microorganisms are not needlessly exposed to sublethal concentration of the biocide, a situation which this report shows breeds development of resistance.

Chlorine releasing compounds

Introduction

Reactive chlorine species are oxidizing agents that can be used to irreversibly damage microorganisms. These species are released in the medium by chlorine releasing agents. The most important types of chlorine releasing agents are sodium hypochlorite, chlorine dioxide, and the N-chloro compounds such as sodium dichloroisocyanurate (NaDCC), with chloramine-T also being used. Of these, sodium hypochlorite (NaOCl, CAS number 7681-52-9), approved as a PT1, PT2, PT3, PT4 and PT5 product in the EU (and under review for PT11 and PT12), also known as household bleach, is the most used chlorine-based disinfectant (13, 15). As such, we will focus on this product for this report. Data pertaining to other chlorine releasing agents was rare, but as the activity of all chlorine releasing agents is due to the release of free chlorine in the medium, insights on the emergence of resistance following the use of one biocidal product of the category should be roughly applicable for other products of the category.

In aqueous solution of NaOCl, chlorine exists as chlorine gas (Cl_2), hypochlorous acid (HOCl), and hypochlorite ion (^{-}OCl) in equilibrium. The higher concentration of HOCl can be found at a pH between 4 and 6. At pH values lower than 4, Cl_2 becomes the predominant chlorine specie. At higher pH values (between 8.5-10), ^{-}OCl becomes the major component of the solution. Among these forms of chlorine, HOCl has the greatest germicidal action (80-fold greater than ^{-}OCl) and determines the activity of diluted NaOCl solution since it is neutrally charged and can easily penetrate the lipid bilayer of the membrane (13). The activity is strongly reduced by the presence of organic load and in general by the presence of particles (215). It is important to note that HOCl is one of the main oxidants produced by neutrophils in the innate immune response and is thus a substance that pathogens have naturally encountered for a long time.

NaOCl and its active ingredient HOCl are widely used for sanitation and disinfection purposes in industrial, hospital, and household settings. For instance, sodium hypochlorite is used in biocides (wiping disinfectants for surfaces with 0.05-0.5%, spraying disinfectants with up to 3%, or skin disinfection with 0.1%). In health care, the substance can be used as a hand scrub (0.01-0.05%), surgical site antiseptic (0.01-0.05%), mucosa and wound antiseptic (0.005-0.01%), surface disinfectant (0.0125-0.05%) and instrument disinfectant (0.0125-0.05%). Sodium hypochlorite is also used for wound antisepsis and antiseptic treatment of burns. It is frequently used in water disinfection, for example, in swimming pools and in the water treatment process (13, 215).

Hypochlorous acid is extremely reactive with various cellular components, as it undergoes a rapid reaction with nucleophilic structures, such as hemes and porphyrins, iron-sulfur proteins, purine and pyrimidine bases, sulfhydryl groups, amines, and amino acids. Due to its neutral charge, it easily penetrates the cell wall and membrane of bacterial cells, leading to damage to membrane proteins responsible for energy transduction and transport, leading to rapid ATP hydrolysis. ATP production, as well as metabolite and protein transport are inhibited. Proteins are fragmented due to the cleavage of peptide bonds, and proteins start to unfold, which leads to the irreversible aggregation of essential bacterial proteins, and consequent bacterial death. HOCl also inhibits protein and DNA synthesis due to its interaction with proteins involved in translation and transcription and causes the dissociation of DNA double-strands. In addition, HOCl reacts with lipids and increases the permeability of the membrane (13).

Sodium hypochlorite is mostly sporicidal, mycobactericidal, bactericidal, fungicidal and is active against certain viruses (215). The use of NaOCl as a disinfectant to clean surfaces and medical equipment has increased during the current SARS-CoV-2 pandemic. The virus can be inactivated by a 0.1% solution of NaOCl. The effectiveness of NaOCl to disinfect several viruses, including Ebola virus, Norwalk virus, murine norovirus, and human immunodeficiency virus (HIV) has also been reported. Enveloped viruses, such as SARS-CoV-2, are inactivated by NaOCl due to its interaction with the viral outer lipid envelope (13).

For bacteria, the highest MIC values have been found in isolates of *E. faecalis*, (3.2%), *E. coli* (1.2%), *Lactobacillus* spp. (0.4%), *L. monocytogenes* (0.78%), *P. aeruginosa* (0.8%), and *S. aureus* (1.6%) (215). These values are higher than the concentration used in some biocidal products, suggesting that some level of resistance might be attained by some bacterial species. For yeasts, the highest MIC recorded is attained by *C. albicans* at 1.6%, although the majority of MIC values for *Candida* spp., *Aspergillus* spp., *Penicillum* spp., *Mucor* spp., *Rhizopus* spp. and *Trichoderma* spp. is around 0.2% (215).

The dramatic increase in use of disinfectant, including bleach, during the SARS-CoV-2 pandemic carries with it the worrisome potential for increased development of microbial resistance to these compounds. In this section we go over the occurrence of emergence of resistance and related mechanisms found in the literature.

Increased resistance to disinfection in biofilms

As is often the case, bacterial cells that are embedded in a biofilm are much more resistant to the action of sodium hypochlorite. One difference is that contrary to other biocidal agents such as chlorhexidine digluconate, sodium hypochlorite is actually able to dissolve the exopolysaccharide matrix of biofilms (216), which gives this biocide a clear advantage in terms of sanitation and sterilization of surface and instruments. For instance, in one study, sodium hypochlorite was better than peracetic acid (both at 0.005%) at eliminating biofilms of different heterotrophic bacteria isolated from a minimally processed vegetables plant. However neither of the biocides were able to completely eradicate the biofilms and significant regrowth was observed for most of the biofilms (217). In this section, we go over occurrences of increased resistance of planktonic microbial cells compared to biofilm cells.

Biofilms of *P. marginalis* grown in maple sap were up to 13.5-fold less susceptible to sodium hypochlorite (OXY CHLOR 12, Atomes) (109). Planktonic cells of isolates of *P. aeruginosa* from two dairies were about 2 to 3-fold more sensitive to sodium hypochlorite than biofilm of these strains when they were in contact with the biocide for 5 min (184). In non-typeable *H. influenzae*, treatment of a dispersed biofilm with 0.0012% sodium hypochlorite led to a 5-$\log_{10}$ reduction in CFU, compared to no reduction in the number of CFU when a non-dispersed biofilm was treated with the same agent, indicating that resistance of this organism in biofilms is largely mediated by the physical properties of the biofilm (132). In 1% sodium hypochlorite (CAS 7681-52-9), biofilms of *S. mutans* was viable up to 30 min on a glass carrier and *S.* Typhimurium up to 45 min on a rubber carrier (22). Elimination in 10 min of *S. aureus* biofilm cells required 10-fold the concentration of sodium hypochlorite than planktonic cells. Exposure to 2% sodium hypochlorite achieved a 7-$\log_{10}$ reduction in CFU and reduced biofilm mass by a factor of 100, but alive *S. aureus* cells remained and regrew on prolonged incubation (218).

E. faecalis cells in mature and old root canal biofilms have higher resistance to 1% sodium hypochlorite compared to the young biofilms. However, 2.5% and 5.25% sodium hypochlorite

caused complete inhibition of the growth of *E. faecalis* biofilm in all stages of development (219).

A. hydrophila biofilms required about 20-fold the concentration of sodium hypochlorite to exhibit similar inactivation kinetics as planktonic cells (220).

Multispecies biofilms (*Acinetobacter calcoaceticus*, *B. cepacia*, *Methylobacterium* sp., *Mycobacterium mucogenicum*, *Sphingomonas capsulata*, and *Staphylococcus* sp. isolated from a model drinking water distribution system) were usually more resistant to inactivation and removal by sodium hypochlorite (Sigma) than single-species biofilms. Total biofilm inactivation was achieved only for *A. calcoaceticus* single-species biofilms and for the multispecies biofilms without *A. calcoaceticus*. Biofilms with all bacteria had the highest resistance to sodium hypochlorite. Thus *A. calcoaceticus* formed single-species biofilms susceptible to sodium hypochlorite, but its presence in multispecies biofilms increased their resistance to disinfection (221).

L. plantarum subsp. *plantarum* JCM 1149 planktonic cells were eliminated by 0.005% sodium hypochlorite, while biofilm populations were stable up to the maximum concentration tested of 0.025% (222). Planktonic cells of *C. albicans* were completely killed off by 0.01% sodium hypochlorite in 5 min, whereas there was only a 4-$\log_{10}$ reduction when biofilms cells were treated in the same manner (223). Sodium hypochlorite used at 1.312% for 4 min had an overall better disinfection efficacy than two quaternary ammonium compounds against biofilms of *S. aureus* (ATCC-6538) and *P. aeruginosa* (ATCC-15442) (77).

Emergence of resistance

Exposure of microbes to sub-inhibitory concentrations of chlorine releasing agents has been extensively covered in the literature. This section sums up the data that was reviewed and provides extra explanation on the cases where resistance is highest and when pre-exposure to chlorine releasing agents is associated with cross-resistance to other antimicrobials.

Stepwise sub-inhibitory exposure of *E. coli* ATCC 12806 (Serotype O124:K72(B17):H) to sodium hypochlorite led to a 2-fold increase in the MIC of the bacterium against this biocide. The adapted strain was able to survive after treatment with 0.06% sodium hypochlorite (50% survival). The adapted strain also developed resistance to 3 antibiotics (amongst 29 tested) as measured by disk diffusion assay: **spectinomycin** (100 µg on disk), **ampicillin-sulbactam** (20 µg on disk) and **nalidixic acid** (30 µg on disk). Sodium hypochlorite-adapted cells also had increased resistance to **sodium nitrate** (224). Exposure of biofilm cells to sublethal concentration of sodium hypochlorite (0.3%) (CAS 7681-52-9) enhanced the biofilm forming ability of *Salmonella* Enteritidis NCTC 13349 (85).

Exposing *E. coli* CMCC44103 to low concentrations of sodium hypochlorite (final chlorine concentration of 0.05%) induced a viable but non-culturable (VBNC) state in which the cells are alive, but cannot be cultivated using standard cultivation technique (225). The VBNC is usually associated with resistance to high doses of antibiotics, increased levels of pH, heat, ethanol, and heavy metals (13). In this state, the bacteria were shown to have reduced metabolic activity and increased persistence to 9 typical antibiotics (the cells remained viable at antibiotic concentrations of 16- to 256-fold MIC). The antibiotics used were ampicillin, gentamicin, polymyxin, ciprofloxacin, terramycin, tetracycline, rifampicin, clarithromycin, and chloromycetin. These viable but non-culturable cells had increased expression of stress-related genes (such as *rpoS, marA, ygfA, relE*) and genes related to antibiotic resistance genes, mainly

efflux pumps (*folA, tolC, acrD, acrF, emrA, macA,* and *macB*). Since these viable but non-culturable cells are not picked up by monitoring technique relying on the culture of bacteria, they might constitute a hidden source of persistent bacteria that evade disinfection and that might ultimately be detrimental to human health (225).

Treatment with low concentrations of sodium hypochlorite was shown to increase the rate of horizontal transfer of antibiotic resistance genes in *E. coli* and *S.* Typhimurium. This process may be due to increased membrane permeability and increased expression of genes involved in the conjugation process. These results suggest that gene transfer induced by sodium hypochlorite exposure might lead to bacterial dissemination and resistance in different environments (13, 226). Chlorination of drinking water (sampled from a drinking water treatment plant in Nanjing, China, chlorination method not communicated) leads to an increase in pathogenicity islands (carrying mobile genetic elements that may contribute to the virulence of the pathogen, including adherence factors, toxins, iron uptake systems, invasion factors and secretion systems) detected by high-throughput sequencing and the concentrations of virulence proteins, such as flagellar motor switch protein (FliG), Clp protease, and inner membrane protein OxaA. The authors attribute this increase in virulence factor to an increased horizontal gene transfer amongst the surviving bacteria after disinfection (227).

Pretreatment of *E. coli* K12 with 0.03% HOCl (the reagent used to obtain HOCl in the solution being sodium hypochlorite) leads to resistance to killing by 10 mM H_2O_2. In the exponential phase, induction of the *oxyR* regulon, an adaptive response to H_2O_2, protected against HOCl exposure (86).

One strain of *L. monocytogenes* serotype 1/2c isolated from a poultry plant had a 2-fold increase in MIC (from 0.025 to 0.05%) when repeatedly exposed to sodium hypochlorite (active chlorine 10%) (Finnish Chemicals, Äetsä, Finland). This sodium hypochlorite-adapted strain also displayed increased resistance (4-fold increase in MIC) towards a **quaternary ammonium compound (n-alkyldimethyl ethylbenzyl ammonium chloride)** (Pointing Chemicals, Huddersfield, UK) (201).

Exposure of different *S. enterica* strains to gradually higher concentration of sodium hypochlorite (Sigma) led to a slight increase in resistance for some of these strains. The maximum tolerable concentration of sodium hypochlorite increases from about 0.04% to 0.1% for *Salmonella* Hadar and 0.15% for *Salmonella* Infantis. Some of the adapted isolates were found to become resistant to different classical antibiotics as assessed by disk diffusion assay. The MIC were not calculated for these antibiotics and the relevant mechanism were not investigated (228). A *S. enterica* serotype Typhimurium strain (S175) isolated from poultry developed a slight increase in resistance to sodium hypochlorite after being grown in sub-inhibitory concentrations of the biocide. The increase was low (the strain was able to grow in 1% sodium hypochlorite (10% active chlorine) (Sigma) compared to 0.6% before adaptation), but was accompanied with an increased biofilm formation ability (229).

Similarly, a methicillin-resistant *S. aureus* strain (MRSA 48a) previously isolated from a poultry hamburger developed a slight increase in resistance to sodium hypochlorite after being grown in sub-inhibitory concentrations of the biocide (MIC went from 0.5% to 0.84% sodium hypochlorite (10% active chlorine) (Sigma)). Growth of the adapted strain in the presence of a sub-inhibitory concentration of sodium hypochlorite led to a 2-fold increase in biofilm formation (measured by the volume of biofilm formed) (230).

Growth of *S. aureus* ATCC 29213 in sub-inhibitory concentration of sodium hypochlorite (CAS 7681-52-9) for extended period of time (0.005% for 72h) led to the development of a mutant that is resistant to **oxacillin**, with a 16-fold increase in the MIC compared to the non-adapted strain, to a value superior to 0.0004%, which is the cutoff for clinical resistance. This increased resistance was not accompanied by an increase in the MIC for sodium hypochlorite (231). This mutant had a loss-of-function mutation in the *gdpP* gene, a phosphodiesterase that regulates gene expression. Loss of function of the GdpP protein has been previously described in association with borderline oxacillin resistance. Transmission electron microscopy also revealed a significantly thickened cell wall, which may be involved in oxacillin resistance (231).

Exposure of *P. aeruginosa* cells to sub-inhibitory levels of sodium hypochlorite (0.0004%) increased the resistance of the bacteria to **ceftazidime, chloramphenicol, and ampicillin** (1.4 to 5.6-fold compared to the control), which was associated with the upregulation of the efflux pump MexEF-OprN (232).

Fifty *Pseudomonas* clinical isolates were exposed to the maximal sub-inhibitory concentration of sodium hypochlorite (Clorox 5.25%, actual concentration used was not communicated) overnight. After this incubation, the MIC against different antibiotics (Piperacillin-tazobactam, ceftazidime, gentamicin, amikacin, ciprofloxacin, meropenem and colistin) was measured. For each of these antibiotics, a significant proportion of the isolated displayed increased resistance. Furthermore, for **colistin, meropenem** and **ceftazidin**, there was at least one isolate that was above the threshold for clinical resistance for *Pseudomonas* according to the Clinical and Laboratory Standards Institute (2016) (202).

Mechanisms of resistance

The response of microorganisms to reactive chlorine species such as sodium hypochlorite is not as well described as the response to ROS. Since hypochlorous acid (the active agent in sodium hypochlorite solutions) is a chemical species the microbes encounter in nature, the response is complex and involves multiple pathways. Moreover, it is not known how each of these pathways contribute to clinical resistance. In this section we summarize the main mechanisms of adaptation to sodium hypochlorite and detail studies that may bring some insight into the mechanism of resistance to this biocide.

In bacteria, hypochlorous acid induces the expression of several detoxifying enzymes, including catalases, peroxidases, and superoxide dismutases. It activates the expression of chaperones, DNA and protein repair systems, methionine sulfoxide reductases (Msrs) and induces changes in the membrane, such as increasing hydrophobicity, reducing permeability, and decreasing the amount of porins (13). In *E. coli*, these transcriptional changes are mostly controlled by three HOCl-specific transcriptional regulators, HypT, RclR, and NemR, as well as by two regulators, OxyR and SoxR, that are involved in ROS response but have also been described to be involved in HOCl resistance in Gram-negative bacteria (13). Other regulators may also be involved, such as SlyA, a transcription factor of *Salmonella* Typhimurium that regulates the expression of genes involved in virulence (*sopD, sopE2, hilA)* and central metabolism (*kgtP, glpA, fruK)* in response to sodium hypochlorite and hydrogen peroxide (94).

One study used RNA-seq to investigate the gene expression response of *P. fluorescens* (ATCC 13525) biofilms when exposed to sub-lethal concentrations of sodium hypochlorite (0.00001%). The biofilms increased transcription of genes encoding peroxide scavenging

enzymes (alkyl hydroperoxide reductase (Ahp) and hydroperoxide resistance protein (Ohr)), oxidative stress repair enzymes (the periplasmic sulfoxide reductase MsrPQ complex), and multidrug efflux (MexEF pumps). Genes involved in amino acid synthesis and energy metabolism were down-regulated following hypochlorite exposure. The authors conclude that *P. fluorescens* biofilms respond to oxidative stress induced by sodium hypochlorite through three targeted genetic mechanisms: (1) active neutralization of oxidizing agents through the Ohr and Ahp complexes; (2) active repair of proteins and membrane residues to manage damage induced by oxidative stress; (3) active removal of chlorine and organic chlorinated molecules resulting from the reaction of sodium hypochlorite and cell constituents through multidrug efflux. They also point out that a general or non-specific stress response may also act to thwart sodium hypochlorite stress, as several genes involved in osmotic, heavy metal-induced, or starvation-induced stress mechanisms were differentially expressed in their assay (233).

Another study found that the most upregulated genes in *P. aeruginosa* in response to HOCl exposure were genes associated with antibiotic resistance, and protein secretion and export systems. The exposure to HOCl also activates virulence systems used to overcome the host immune system, including the induction of pyocyanin production by *P. aeruginosa* and the activation of the type 3 secretion system (T3SS) (13, 234). The fluoroquinolone-, chloramphenicol-, and trimethoprim-exporting MexEF-OprN efflux pump was also induced by treatment with HOCl and found to be necessary for resistance to the chemical. Since MexEF-OprN can expel products other than antibiotics the authors propose that MexEF-OprN might expel toxic by-products of HOCl reactions with cellular components (234). The MexEF-OprN efflux pump is thus involved in both cross-resistance to antibiotic (by increasing the efflux of said antibiotics) and increased resistance to sodium hypochlorite by transporting toxic species generated by hypochlorous acid outside of the cell (232, 234).

The exposure of *Xanthomonas campestris* pathovar *campestris* (ATCC 33913) to sublethal concentrations (0.0625 and 0.0313% (m/v) NaOCl for 15 min) of a sodium hypochlorite (NaOCl, 12.5% (m/v), Ajax Finechem) solution induced the expression of genes that encode peroxide scavenging enzymes within the OxyR and OhrR regulons. *oxyR*, *katA*, *katG*, *ahpC*, and *ohr* were shown to contribute to protection against NaOCl killing. Treating the bacteria with a low concentration of NaOCl (0.625%) resulted in the adaptive protection from NaOCl killing and also provided cross-protection from H_2O_2 killing. The authors conclude that the results suggest that the toxicity of NaOCl is partially mediated by the generation of peroxides and other reactive oxygen species that are removed by primary peroxide scavenging enzymes, such as catalases and AhpC, as a part of an overall strategy that protects the bacteria from the lethal effects of NaOCl (235).

Conclusion

Chlorine releasing compounds, as exemplified by sodium hypochlorite or bleach, are one of the most ubiquitous biocides, used widely both in households and clinical settings. Sodium hypochlorite has been used as a disinfectant since the 1820's (236) and no significant reports of microbial resistance have emerged since then. Low level increases in MIC after exposure to low concentrations of the biocide have been documented in *E. coli, S. enterica, L. monocytogenes* and *S. aureus*, but are probably not a worrisome threat for human health. Similarly, no significant outbreaks have been linked to resistance to sodium hypochlorite disinfection. Biofilms constitute a more resistant reservoir that can lead to dissemination of microorganisms after disinfection procedures, but sodium hypochlorite has the added advantage

compared to other biocides of actually dissolving the exopolysaccharide matrix of the biofilm, thereby helping to prevent regrowth of the biofilm.

There are a few reports of low-level cross-resistance to other antimicrobials after exposure to low concentrations of sodium hypochlorite and some of these reports were considered above the threshold for clinical resistance. The substances concerned by cross-resistance are: sodium nitrate, hydrogen peroxide, nalidixic acid, ampicillin-sulbactam, a quaternary ammonium compound, oxacillin, ceftazidime, chloramphenicol, ampicillin, colistin, meropenem and ceftazidim. Again, these reports are few considering the wide usage and the length of time that sodium hypochlorite has been used as a biocide, and there was no report of a link between cross-resistance to antibiotics and actual clinical hazard. The mode of action of sodium hypochlorite is largely non-specific. The mechanism of resistance to the biocide involves several transcriptional regulators and no single gene has been linked to sodium hypochlorite resistance, although an efflux pump that is expressed in presence of sodium hypochlorite has been linked both to increased resistance to the biocide and low-level cross-resistance to some antibiotics.

All in all, if recommended guidelines for the use of chlorine releasing compounds are followed so as to limit exposure of microorganisms to sublethal dose of biocide, the risk for the development of resistance and cross-resistance should be limited.

Lactic acid and other weak acids

Introduction

Weak organic acids such as acetic acid (the main ingredient of vinegar) and lactic acid have been used for centuries to preserve food and decontaminate infected environments (237). Nowadays, in addition to their role as food preservative, the use of acetic, sorbic and lactic acid as disinfectants seems to be mostly in the decontamination of meat carcass in the food industry. Acetic acid is authorized in the EU as part of Annex 1 of the Biocidal Products Regulation as a low-risk substance, but is not part of the EU review program for use as a biocide. Lactic acid (CAS number 79-33-4) is authorized as a PT1, PT2, PT3, PT4 and under review for PT6 in the EU.

Despite their long-standing widespread use, the antimicrobial mode of action of weak acids is still not fully understood. It is generally agreed that the biocidal activity of weak acids is related to their lipid permeability. Weak acids can be either charged or uncharged, depending on the pH of the environment. The uncharged form of the weak acid is lipid permeable and can therefore diffuse into the cytoplasm of microbial cells. Since most microorganisms maintain a pH gradient across their cytoplasmic membranes, with the inside being more alkaline than the outside, the result is the accumulation of high levels of the charged weak acid in the cytoplasm (238). The antimicrobial activity of weak acids has been attributed to effects on intracellular pH (pHi). When the uncharged weak acid dissociates inside the cytoplasm, the weak acid anion starts to accumulate and protons are released, leading to a drop in pHi. However, bacterial cultures grown in the presence of different weak acids can grow at the same rate even when they have significantly different pHi values, suggesting that a lower pHi cannot be the sole determinant of bactericidal activity (238). The inhibitory effect of weak organic acid might be due to membrane perturbations that result from acids interacting with the membrane and accumulation of the weak acid anion inside the cytoplasm, which would lead to osmotic stress and perturbation of certain enzymatic metabolic reactions (238).

Acetic acid and lactic acid mostly have a fungistatic and bacteriostatic effect, inhibiting the outgrowth of these organisms, although depending on the concentration, pH and time of treatment, a bactericidal activity can be observed (239). Interestingly, acetic acid at 6% had a significant bactericidal effect on mycobacteria in solution (240), and sorbic acid also inhibits the outgrowth and germination of bacterial spores (241).

In this section we summarize the data available on the development of resistance to weak organic acids such as acetic acid or lactic acid and the mechanisms of adaption of microorganisms to these substances.

Emergence of resistance

Although the adaptation of microorganisms to weak organic acids has been largely investigated, very few data is available on the development of resistance when these products are used as a disinfectant or biocide. In this section we detail the study that have found increased resistance after exposure to acetic and lactic acid.

It has been known for decades that bacteria can adapt to acid stress. In 1989, Goodson & Rowbury (242) showed that strains of *E. coli* (1829 ColV, I-K94) grown at pH 7.0 failed to grow after relatively short periods of exposure to pH 3.0 or 3.5**,** but after initial growth in medium at pH 5.0**,** they were almost unaffected by exposure to the low pH values. In 1990,

Foster & Hall (243) showed that logarithmically grown cells (pH 7.6) that were shifted to mild acid (pH 5.8) for one doubling as an adaptive procedure were 100- to 1000-fold more resistant to subsequent strong acid challenge (pH 3.3) than were unadapted cells shifted directly from pH 7.6 to 3.3. Similar data were obtained for *L. monocytogenes* (244, 245). This phenomenon was called acid tolerance response (ATR).

It is possible that this acid tolerant response might protect bacteria from killing by organic acids. *E. coli* (1829 ColV, I-K94) cells that were grown at pH 5 had increased survival when exposed to lactic, propionic, benzoic, sorbic, trans-cinnamic and acetic acid at pH 3.5 compared to *E. coli* cells that were grown at pH 7 (246). There was complete killing of cells when *S.* Typhimurium (*UK1 x3761)* was exposed to butyric, acetic or propionic acid at pH 4.4. But when cells were pre-adapted for 1h to pH 4.4, there was a drastic survival of at least 4-$\log_{10}$ at 80 min of treatment (247).

Van Netten *et al.* (248) found that acid-adapted *E. coli* O157:H7, *S.* Typhimurium*, S. aureus,* and *C. jejuni* that contaminate skin surface of pork belly cuts were slightly more resistant to decontamination with 2% lactic acid than non-adapted strains, but were still susceptible to killing and did not cause an extra health hazard. Using a mixture of acetic acid (0.8%) and lactic acid (0.2%) (Macklin, Shanghai) to disinfect lettuce led to a significant decrease of the abundance of *Massilia* spp. and *Alkanindiges* spp. but there was a marked increase in *Escherichia-Shigella* abundance indicating that acid disinfection altered the microbial ecology to stimulate *Escherichia-Shigella* growth (249). Four rifampicin resistant (10%) derivatives of *E. coli* O157:H7 strains ATCC 43895, ATCC 43889, ATCC 51658 and EO139 that were exposed to lactic acid washing solutions for 24h had enhanced survival for up to 180 min of acid challenge at pH 3.5 compared to cells that were not exposed to lactic acid washing solutions (250). Growth in acidic medium (acidified with hydrochloric acid) was associated with increases in the MIC to amikacin, ceftriaxone and nalidixic acid (*E. coli*), gentamicin and erythromycin (*S. aureus*) and amikacin, ceftriaxone and trimethoprim (*S.* Typhimurium) (251). However, it is not known whether low pH obtained with the addition of organic acids such as lactic acid would yield the same results, since the antimicrobial effect of weak organic acids is thought to be due to the accumulation of the anion inside the cell. *E. coli* O157:H7 incubated in acidic washings of sublethal pH (4.89-5.22) became acid-habituated and more tolerant to acid conditions. The ATR of the pathogen inoculated into washings was enhanced when cells were previously acid-adapted and incubated at 4°C. Acid-adapted cells were consistently more resistant to lactic acid washings throughout the experiment. Acid-adapted *E. coli* O157:H7 may become resistant to subsequent lactic acid exposure after storage at 4°C (252). Other studies have found that the expression or maintenance of acid tolerance by *E. coli* O157: H7 strain ATCC 43895 is enhanced after exposure to organic acids such as lactic acid or acetic acid (253, 254).

L. monocytogenes strain Scott A (serotype 4b, lineage I, epidemic strain, human isolate) that was acid-adapted during planktonic growth (in a growth medium that had a decreasing pH during incubation, with a final value of 4.6 at the end of the incubation) was more resistant as biofilm when treated with lactic acid (0.05%, corresponding to a pH of the solution of 3). The non-acid-adapted control biofilm had about 1-$\log_{10}$ reduction of sessile cells, compared to less than 0.5-$\log_{10}$ reduction for the acid-adapted cells (255). One study compared the ability of multi-antibiotic-resistant (n = 8), antibiotic-resistant (n = 18) and antibiotic susceptible (n = 11) *L. monocytogenes* strains from food and clinical origin to survive to lactic acid stress (1% v/v DL- lactic acid (Fluka, Neu-Ulm, Germany) to a final concentration of 134 mM, to obtain a final pH 3.5). It was found that antibiotic sensitive strains presented mean values of logarithmic

reduction significantly higher than antibiotic resistant or multi antibiotic-resistant strains. When evaluating separately the antibiotic resistance groups, strains resistant to erythromycin, ciprofloxacin and nitrofurantoin were significantly more resistant to the lactic acid stress than the antibiotic sensitive isolates. Both food and clinical strains resistant to two or three antibiotics were significantly less susceptible to lactic acid. Whether this cross-resistance can be attributed to efflux remains to be investigated (256). Repetitive inactivation with lactic acid of a cocktail of 4 *L. monocytogenes* strains yielded a culture of higher resistance in comparison to the parental culture. The lactic acid-adapted strain (originating from LMG 13305, isolated from soft cheese) had a 1.28-$\log_{10}$ reduction when treated with 2.5% lactic acid at pH 3.5, compared to 2.15-$\log_{10}$ reduction for the parental strain (257).

The EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP), in a report titled "Evaluation of the safety and efficacy of the organic acids lactic and acetic acids to reduce microbiological surface contamination on pork carcasses and pork cuts" made the following conclusion on the potential emergence of reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials linked to the use of lactic and acetic acid : "There is some evidence that repeated exposure to lactic acid can select for reduced susceptibility to the same substance. This may be favored by an increased level of lactic acid on the meat surface compared to that naturally present and/or to the associated low pH of treated meat surfaces. However, under GHP [Good Hygienic Practices] the Panel did not consider this a significant issue. There is no evidence suggesting the promotion of a horizontally transferable reduced susceptibility to lactic or acetic acid or resistance to therapeutic antimicrobials as a result of exposure to lactic or acetic acid. Considering the extensive natural presence of lactic and acetic acid, including in feed and food, the possibility of development of resistance to therapeutic antimicrobials is also unlikely to be a significant issue." (258)

Mechanism of adaptation

The EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) summarizes the mechanism of adaptation to acidic conditions: "This involves a combination of constitutive and inducible strategies including: (1) the direct removal of protons from the cell using proton pumps; (2) changes in the composition of the cell membrane (e.g. by increasing the concentration of cyclopropane fatty acids and/or blocking outer membrane porins by binding polyphosphate or cadaverine); (3) the alkalization of the external environment by switching metabolic systems so that less acid is produced (e.g. using ribose, arabinose and fructose as the carbon sources, all of which result in less acid production as compared to glucose metabolism); (4) the direct consumption of intracellular protons using the hydrogen-gas-producing formate hydrogen lyase (FHL) complex and the pyridoxal-5-phosphate (PLP)-dependent amino acid decarboxylase AR systems and (5) the production of general shock proteins and chaperones." (258)

The mechanism of adaptation to weak organic acid is thus complex and involves many different pathways. To the best of our knowledge, no single gene that is transferable by horizontal gene transfer can confer resistance to weak organic acids.

Conclusion

Organic acids, and lactic acid in particular, have been used as a means to preserve food for centuries. They are also a natural part of bacterial metabolism. There are some reports in the literature that some strains of *E. coli*, *S.* Typhimurium*, S. aureus, C. jejuni* and *L. monocytogenes* can become resistant to acidic conditions, and thus more resistant to

disinfection by organic acids such as lactic acid. There is one report that antibiotic resistant bacteria may also be more resistant to disinfection by lactic acid, which could potentially result in increased exposure of humans to antibiotic-resistant bacteria. However, these reports are scarce, especially considering the widespread use of organic acid as biocides, and their impact on public health are largely unknown. Because bacteria have been in widespread contact with these substances for such a long time, it is unlikely that they can develop worrisome resistance in the future. If they could have done it, it is probable that they would already have done it. The same is true for cross-resistance to antibiotics. There is no actual report of cross-resistance to other antimicrobials following the use of weak organic acid and **there is thus insufficient evidence to suggest that the use of organic acids can lead to antibiotic or biocide resistance**. The precautionary principle should still apply, and care should be taken to ensure that microorganisms are exposed as little as possible to sublethal concentrations of the biocide.

Triclosan

Introduction

Triclosan (CAS number 3380-34-5) is a chlorinated biphenyl antimicrobial agent that is widely used in household products, including cosmetics and antimicrobial soaps (259). **Important to note, triclosan is not approved in the EU as a biocide and its presence in biocidal products has been banned since 2017** (ECHA). It can still be found under certain conditions as a preservative in cosmetics. At low concentration, it inhibits fatty acid biosynthesis by targeting a highly conserved enoyl-acyl carrier protein reductase (ENR, *fab* genes) (260, 261). There are various mechanisms that are known to confer triclosan resistance in bacteria (262, 263), including the overexpression of ENR (264), the presence of mutated and/or triclosan-resistant ENR (265, 266); and the upregulation of efflux pumps (267-269). Triclosan exhibits particular activity against Gram-positive bacteria but is also effective against Gram-negative bacteria and yeast (15, 259).

Emergence of resistance

Several studies have found that the presence of triclosan in the medium exerts selective pressure and induces co- or cross-resistance to other antibiotics (267, 270-274). Here we detail the recent research in the development of resistance to triclosan in different bacterial species, both in laboratory settings and environmental samples.

Initial studies found that *E. coli* could become resistant to triclosan through either mutations in the *fabI* gene or its overexpression (261). A single mutation in the *fabI* gene is responsible for a 400-fold increase in the MIC of *E. coli* against triclosan (275). While one study noted no cross-resistance to other antibiotics in triclosan-resistant strains (and even an increased susceptibility to aminoglucosides) (276), other works on the subject seem to indicate that cross-resistance is not only possible, but widespread. In one study, different *E. coli* strains that were exposed to sub-lethal concentrations of triclosan demonstrated cross-resistance to **chloramphenicol, trimethoprim, tetracycline, amoxicillin, amoxicillin/clavulanic acid, trimethoprim, benzalkonium chloride** and **chlorhexidine** (277). The authors suggested that the development of cross-resistance was due to multidrug efflux pumps and mutations in the *fabI* gene (277). Low dose treatment for 24h also rendered *E. coli* susceptible to tobramycin (273).

It was also reported that clinically relevant concentrations of triclosan increased *E. coli* and MRSA resistance to bactericidal antibiotics as much as 10,000-fold *in vitro* and reduced antibiotic efficacy up to 100-fold in a mouse urinary tract infection model. Genetic analysis indicated that triclosan-mediated antibiotic resistance requires ppGpp whose accumulation has been repeatedly associated with antibiotic tolerance and persistence (278).

A recent study found that among 200 *E. coli* isolates from urine samples, 2.5% were resistant to triclosan and exhibited multi-drug resistant phenotypes (274). Furthermore, they showed that triclosan-susceptible strains could become triclosan resistant after being exposed to sub-inhibitory concentrations of the biocide. This resistance phenotype was also associated with cross-resistance to **ciprofloxacin, tobramycin, levofloxacin** and **cefepime**. The multi-drug resistant or cross-resistance phenotype was associated with elevated expression of efflux pump genes (274).

A high-throughput genomic approach in *E. coli* identified novel genes as being sensitive to triclosan in addition to the fatty acid synthesis genes and efflux pumps. These include genes involved in barrier function, small molecule uptake, and integrity of transcription and translation (263).

In contrast to *E. coli, P. aeruginosa* is naturally unsusceptible to triclosan. This resistance has been demonstrated to be due to at least two mechanisms: (i) *P. aeruginosa* has two triclosan-resistant enoyl-ACP reductase isozymes (*fabI* and *fabV*) (279) and (ii) it expresses efflux pumps (the MexAB-OprM system) (267). Of note, a fatal epidemy in an oncohematology unit was driven by a *P. aeruginosa*-contaminated triclosan soap dispenser (280). Worryingly, the epidemic strain adapted to triclosan became more resistant (2-fold the MIC value) to antibiotics that are typically exported by efflux pump, namely **tetracycline, ciprofloxacin, amikacin, levofloxacin, carbenicillin** and **chloramphenicol** (281).

There are several ways that *Staphylococcus* species can develop triclosan resistance, including active- and non-active-site mutations in FabI (260, 265, 282), formation of small-colony variants (283), horizontal gene transfer (284), and promoter region mutations to increase FabI expression (285). Concerning cross-resistance to other antibiotics, exposure of MRSA to a sub-lethal concentration of triclosan lead to resistance to high doses of **vancomycin** (0.005%) (278). Exposure to triclosan also led *S. aureus* to become resistant to **ciprofloxacin and ampicillin** (286).

Similar to *E. coli, Salmonella* can become resistant to triclosan through different mechanisms, including mutations in *fabI, fabI* overexpression (287), and efflux pumps (288, 289). Interestingly, while mutations in *fabI* were sufficient to attain medium level of resistance in *S.* Typhimurium*,* high level resistance was found to require sigma factor mutations in addition to a *fabI* mutation (290). It would seem that *Salmonella* resistance to triclosan is relatively rare in the environment. In one study, around 20% of *Salmonella* strains isolated from hen eggshells were found to have increased MIC to triclosan compared to the wild-type. There was a correlation between the resistance to triclosan and resistance to the antibiotics **cefotaxime, ceftazidime, ciprofloxacin** and **nalidixic acid** (291). In another study, over 400 animal and human isolates of non-typhoidal *Salmonella* were screened for decreased susceptibility to triclosan and a panel of antibiotics. The prevalence of decreased susceptibility to triclosan was around 4% (288). This may be explained by the lower growth rate sometimes observed in triclosan-adapted strains (287). Of note, 56% of the isolates with decreased triclosan susceptibility, were multidrug-resistant (MDR) compared with 12% of triclosan-sensitive isolates (288), indicating that cross-resistance to antibiotics is relatively likely to occur. Interestingly, as was the case with *E. coli,* triclosan-adapted strains of *Salmonella* were more susceptible to aminoglycosides (287).

Resistance of *Acinetobacter* to triclosan is associated with mutations in *fabI,* overexpression of *fabI* and the overexpression of drug efflux pump (269, 271, 292, 293). Out of 626 clinical isolates isolated between 2016 and 2017, 2.7% were found to be resistant to triclosan (269). The same rate was found in a clinical study with isolates collected between 2004 and 2005 (294), indicating a relatively rare occurrence in clinical settings. In this same study, the resistance rates of triclosan-resistant isolates to **imipenem, levofloxacin, amikacin** and **tetracycline** were higher than those of triclosan-sensitive isolates (294). A triclosan-adapted strain was shown to exhibit slightly elevated resistance to multiple antibiotics, including an 8-fold increase in MIC to **piperacillin** and 4-fold to **doxycycline** (271).

Resistance mechanisms to *Campylobacter* species might include efflux pumps and changes in outer membrane proteins (295, 296). Triclosan-adapted strains developed increased resistance to erythromycin and ciprofloxacin in laboratory conditions (295). Triclosan resistance was also correlated with cross resistance with other drugs in a study that examined 443 *Campylobacter* isolates from humans and animals (297). While *Campylobacter* resistance can occur in the laboratory setting, a study evaluating 40 patients using 0.3% triclosan toothpaste over 5 years found no increase in *Campylobacter* resistance (298). In contrast, amongst 111 *Campylobacter coli* strains obtained from 1998 to 1999 and 2015 from market age pigs and pork chops, all were found to be triclosan-resistant (299).

There are no functional studies that analyzed the mechanism of resistance of *Enterococcus* to triclosan, but it is expected to rely on genetic changes to *fabI* and efflux pumps. Environmental studies collecting a few to hundreds of isolates found no or low-level resistance to triclosan and low occurrence of cross-resistance between triclosan and other drugs in *Enteroccocus* (300-304).

Triclosan resistance and cross-resistance also occur in other bacterial species. In one study, triclosan-resistant mutants of *S. maltophilia* were isolated and found to also have increased resistance to **tetracycline, chloramphenicol** and **ciprofloxacin**. All triclosan-resistant mutants had increased expression of the *smeDEF* multidrug efflux pump (305).

M. tuberculosis is intrinsically resistant to triclosan, while *M. smegmatis* is not. In the latter, resistance can be acquired through *fabI* gene mutation. Indeed, the four residues in *M. smegmatis* InhA which influence triclosan resistance, S94, M103, A124, and M161, are conserved in *M. tuberculosis*, which explains the resistance phenotype of this strain (266). To the best of our knowledge, there are no studies that evaluated cross-resistance with other drugs between treatment of *Mycobacterium* with triclosan.

A study monitoring two wastewater influents in urban and rural communities in the US over a period of 21 months was able to isolate triclosan-resistant bacteria from many different taxa, including *Pseudomonadaceae* (83,3%) and *Enterobacteriaceae* (5.2%). In addition, many of the isolates are of clinical relevance, including genera of multidrug-resistant Gram-negative bacilli (MDR-GNB), considered a high-priority clinical dilemma. Organisms include *Aeromonas* spp., *Serratia* spp., *Burkholderia* spp., and *Klebsiella* spp. The triclosan-resistant isolates were tested for resistance to 13 antibiotics (clindamycin, ampicillin, tetracycline, azithromycin, nitrofurantoin, amoxicillin, erythromycin, trimethoprim, azithromycin, chloramphenicol, gentamycin, ciprofloxacin and cefotaxime), and all were found to be resistant to at least 2 antibiotics, while a few were resistant to all 13 (0.7% of the isolates tested). Resistance to clindamycin, a lincosamide antibiotic, and ampicillin, a β-lactam antibiotic, was observed in nearly all tested isolates. Worryingly, 9% of the isolates were found to be resistant to colistin, a last-resort antibiotic (272).

In another study, triclosan-resistant bacteria were isolated at the effluent of a wastewater treatment plant in South Africa. These bacterial species are resistant to high concentrations of triclosan and were able to grow in the presence of triclosan up to concentrations of 0.00005%. Five main genera were identified, namely *Bacillus, Paenibacillus, Pseudomonas, Brevibacillus* and *Enterococcus.* Antibiotic resistance patterns observed indicated that in most cases, resistance to selected antibiotics (erythromycin, vancomycin, penicillin G) is increased in the presence of high concentrations (0.00005% to 0.0001%) of triclosan (270).

A Genome-wide *in silico* analysis was performed to define the distribution of triclosan-resistant determinants in major pathogens and revealed that potential triclosan resistance determinants were abundant among the majority of human-associated pathogens (79%) and soil-borne plant pathogenic bacteria (98%). These included a variety of enoyl-acyl carrier protein reductase (ENRs) homologues, AcrB efflux pumps, and ENR substitutions. A microbiome analysis revealed that pathogenic genera with intrinsic triclosan-resistant determinants exist in triclosan contaminated environments, indicating that triclosan may not be as effective against the majority of bacterial pathogens as previously presumed (262).

Conclusion

In conclusion, there is a wealth of data that shows that bacteria such as *E. coli, P. aeruginosa Staphylococcus* spp., *Salmonella* spp., *Acinetobacter* spp., *Campylobacter* spp., *Enterococcus* spp., and some mycobacteria readily develop resistance to triclosan. More information on the emergence of resistance for these species and others can be found in the main body of the report.

There are many reports of different bacterial isolates developing cross-resistance to other biocidal products or antibiotics after exposure to triclosan. Table 5 is a summary of the data that was reported in this literature review. More information on cross-resistance following the use of triclosan can be found in the main body of this report.

Table 5 - reports of cross-resistance following exposure to triclosan

Organism	Antimicrobial
Escherichia	Chloramphenicol, trimethoprim, tetracycline, amoxicillin, amoxicillin/clavulanic acid, trimethoprim, benzalkonium chloride, chlorhexidine, ciprofloxacin, tobramycin, levofloxacin and cefepime
Pseudomonas	Tetracycline, ciprofloxacin, amikacin, levofloxacin, carbenicillin, chloramphenicol
Staphylococcus	Vancomycin, ciprofloxacin, ampicillin
Salmonella	Cefotaxine, ceftazidime, ciprofloxacin, nalidixic acid, aminoglycosides
Acinetobacter	Imipenem, levofloxacin, amikacin, tetracycline, piperacillin, doxycycline
Stenotrophomonas	Tetracyclin, chloramphenicol, ciprofloxacin

The mechanisms of resistance are diverse variations of mutations in *fabI, fabI* overexpression and efflux pumps, which is coherent with the vast number of cross-resistance that were reported.

Triclosan might have adverse effects for human health (306) and it has been shown that human absorption of triclosan leads to changes in the microbiome population and size (307, 308), indicating that resistance and cross-resistance could develop in the human body. Considering in addition its chemical properties of bioaccumulation, resistance to degradation and toxic byproducts (309), the decision to ban triclosan in the EU seems justified.

Other biocidal substances

Major biocidal products for in-depth analysis of the potential emergence of resistance after use have been reviewed. In the following section, we summarize the findings on other biocidal products that were not discussed so far. An important resource was a previous report by the Panel on Microbial Ecology of the Norwegian Scientific Committee for Food Safety, which is a literature review conducted in 2016 with the following title: "Antimicrobial resistance due to the use of biocides and heavy metals: a literature review" (24). We also provide further information from additional sources.

The panel found no significant reports of resistance following the use of anilides compounds, such as salicylanilide and triclocarban, iodine and bromine releasing agents, diamidines such as propamidine and dibromopropamidine, quinoline and isoquinoline derivatives, derivatives of 1,3-dioxane, derivatives of imidazole, isothiazolones, derivatives of hexamine, terpenes, and vapour-phase disinfectants such as ethylene oxide, propylene oxide, methyl bromide and ozone (24). To be noted that the lack of report on antimicrobial resistance does not necessarily indicate that there is no actual resistance in practice, as it could be the consequence of a lack of research on the subject. Similarly, this Norwegian report was based on several review articles and not research papers, so the lack of report on resistance to certain antimicrobials could be the result of a lack of review articles based on research papers that document the subject.

The panel found limited information on resistance to antimicrobial dyes such as acridine, triphenylmethane and quinones. It was found that MRSA and MRSE strains that contain *qac* gene were more resistant against acridine and triphenylmethane, possibly due to an efficient efflux system in the resistant strains. Because the area of applications of this class of antimicrobial molecules is limited (24), this is unlikely to be a potential hazard for public health.

Povidone iodine, also called Betadine, is a disinfectant that is mostly used for hand hygiene and as a skin antiseptic. Adaptative resistance following exposure to sub-inhibitory concentrations of the biocide is a rare occurrence, and no cross-resistance to other antimicrobials has been reported yet. *P. aeruginosa* infecting wounds were found to be resistant, but without epidemiological cut off values, it is hard to assess whether this resistance emerged following the use of the biocide, or if the *Pseudomonas* isolates were intrinsically resistant in the first place (310). The use of this biocidal product seems to be low risk for public health.

Kampf (311) has reviewed the data regarding the development of resistance following the use of polyhexanide (PHMB), a cationic biguanide polymer. Although this biocide has similarities with chlorhexidine (also a biguanide), its mode of action is completely different: it enters bacterial cells and binds DNA, leading to cell division arrest and chromosomal condensation (312). No adaptative response was found in most bacterial strains or isolates, although selected strains or isolates revealed somewhat strong MIC changes such as *A. proteolyticus* (16-fold), *E. faecalis* and *S. aureus* (8-fold), *S. capitis* (5.5-fold) and *S. epidermidis* (4.8-fold). No clinical resistance has been reported yet, despite many years of use in many fields. No cross-resistance to other biocidal products or antibiotics has been reported yet. Low-level exposure of MRSA to PHMB increases the MIC value against PHMB but not chlorhexidine.

Silver is the main heavy metal used that belong to category PT1 and PT2. The Norwegian review (24) reports molecular and genetic evidence of silver resistance in *E. cloacae* isolated from skin wounds and medical devices. The *sil* system is the genetic element conferring resistance that is mostly studied. Resistance may be encoded on plasmid or on the chromosome. A recent study analyzing isolates from wounds and burns found that 13% of the isolates were silver-resistant (*K. pneumoniae* (n=7), *S. aureus* (n=4), *E. coli* (n=2), *E. cloacae* (n=2), *P. aeruginosa* (n=2)). The study highlights the lack of research and standardized testing on bacterial silver resistance (313). Silver nanoparticles is a new technology that has been successfully applied in various antimicrobial strategies and household products. One study reports that *E. coli* 013, *P aeruginosa* CCM 3955 and *E. coli* CCM 3954 can develop resistance to silver nanoparticles after repeated exposure. The resistance arises from the production of the adhesive flagellum protein flagellin, which triggers the aggregation of the nanoparticles. This resistance seems to evolve through phenotypic adaptation and no genetic changes (314). The mechanisms surrounding silver resistance should be better understood to determine whether its use as a biocide has the potential for cross-resistance to antibiotics and is a risk for public health.

General conclusions

Does the use of biocidal products in the clinical setting lead to the emergence of resistance to antimicrobials (including antibiotics)?

The short answer is yes. The long answer is that there is a large amount of data that supports a role for biocidal products in the emergence of resistance to antimicrobials, but the importance of this role largely depends on the type of biocidal product used, the microorganism affected and the method and setting in which the biocidal product was used. These specificities are addressed in the following sections by answering questions that cover the objectives of the review.

What are the active biocidal substances that lead to antimicrobial resistance?

Concerning the biocidal products used, it would seem that, as a general rule, the more specific the target, the easiest it is for microorganisms to develop resistance. The obvious example is the extremely large amount of data that documents the emergence of resistance following the use of triclosan, one of the only biocides that has a single specific target. Since most other biocidal products all have non-specific targets, it is hard to establish a quantitative ranking in order of likeliness of development of resistance. In addition, this report is not a risk assessment but a literature review that analyzes the available information, which may be lacking in some respects. We thus propose a qualitative ranking (See Table 6 for a summary):

- There are few reports of resistance following the use of **alcohols, hydrogen peroxide, peracetic acid and weak organic acids**. It is unsurprising given that these compounds are abundant in nature (or are a mixture of compounds that are abundant in nature), have been in contact with microorganisms for a long time and do not easily accumulate in the environment (there is thus less chance of developing resistance in environmental settings). We consider that using these active substances constitutes a **highly unlikely risk** for the development of resistance to antimicrobials.

- There are only a few reports on the development of resistance and cross-resistance to antibiotics following the use of reactive chlorine species. These biocidal products are often used in low concentrations for a large amount of time, which may promote the development of resistance and cross-resistance, as well as increase horizontal gene transfer. Aldehyde-based compounds are not associated with cross-resistance, but they are associated with resistance that led to small-scale outbreaks. We thus consider using **reactive chlorine species and aldehyde-based compounds** an **unlikely risk** for the development of resistance and cross-resistance.

- There is a large amount of evidence characterizing the development of resistance and cross-resistance following the use of **quaternary ammonium compounds and chlorhexidine**, including cross-resistance to the last resort antibiotic colistin. The biocidal products have a tendency to accumulate in the environment, and microorganisms in contact with low concentrations of the products have been demonstrated to develop resistance and cross-resistance. These products often lead to the overexpression of efflux pump which may confer resistance to multiple antimicrobials. We consider the use of these biocidal products a **likely risk** for the development of resistance.

- Finally, triclosan, with its single target, has a very large amount of resistance and cross-resistance reported, mostly through the increased expression of efflux pumps and mutations in the *fabI* fatty acid biosynthesis gene. We consider the use of **triclosan, or any potential biocide with a single specific target**, a **highly likely risk** for the development of resistance. It is therefore appropriate that triclosan is not approved for use in the EU.

Table 6 - Qualitative risk ranking

Alcohols, hydrogen peroxide, peracetic acid and weak organic acids	Highly unlikely
Reactive chlorine species, aldehyde-based biocides	Unlikely
Chlorhexidine, quaternary ammonium compounds	Likely
Triclosan	Highly likely

Again, while this ranking is based on the data gathered in this report, it is not based on a quantitative risk assessment and it is bound to evolve, as microorganisms are living entities that will adapt to changing environmental conditions. There are many uncertainties that could also influence this ranking. A more quantitative approach could be undertaken in the future if we address some of the gaps in knowledge and recommendations that we discuss in later sections.

What are the microorganisms that develop antimicrobial resistance following exposure to biocidal products?

A variety of species seems to be affected: Gram-positive and Gram-negative bacteria, sporulating bacteria, mycobacteria and yeasts. Obviously, the type of microorganism affected depends on the biocidal product used. For instance, mycobacteria have developed resistance to disinfection by aldehyde-based products that has led to small outbreaks in the clinical setting. There were many reports on the development of resistance following the use of chlorhexidine in both Gram-positive and Gram-negative bacteria, but Gram-negative were ultimately resistant to much higher concentrations of the substance than Gram-positive bacteria. In general, Gram-negative bacteria seem to have a higher propensity for the development of resistance following the use of biocidal products than other microorganisms.

Which substances (antibiotics and other antimicrobials) are subject to resistance (cross-resistance or not) as a result of the use of biocidal active substances?

As stated previously, all biocidal products analyzed in this report may lead to some level of resistance to themselves, although not all these biocidal substances may lead to clinical resistance that has implications on human health. The biocidal substances that we consider risky are triclosan, chlorhexidine, quaternary ammonium compounds and, to a much lesser extent, reactive chlorine species and aldehyde-based disinfectants.

However, the use of biocidal products sometimes also leads to the emergence of resistance to antibiotics and other antimicrobials. In this report, we found many such occurrences where resistance to one biocidal product led to resistance to a single or multiple antibiotics. Again, in most instances, the resistance was not clinically relevant, but in some cases, it was enough to be potentially detrimental to public health. Of particular concern, there are reports of colistin (a last resort antibiotic) resistance in some bacterial species following the use of chlorhexidine and quaternary ammonium compounds. The use of triclosan, chlorhexidine and quaternary ammonium compounds may also lead to the resistance of many other antimicrobials, including

antibiotics such as tetracycline, vancomycin, chloramphenicol, ciprofloxacin, imipenem and colistin. More details on all the occurrences of cross-resistance to other biocidal products and to antibiotics are available in the main body of this report.

What are the practices that lead to the emergence of antimicrobial resistance in the hospital setting?

This is a difficult question to answer, as the vast majority of studies look at resistance to biocidal products *in vitro*, and study that analyze real-world situations in the clinical practice, including detailing the uses that may result in resistance to antimicrobial are rare. That being said the data that is gathered in this report provides insights on some practices that may lead to resistance to antimicrobials.

There is a very large number of study reporting that exposure to sub-inhibitory concentrations of a biocidal active substance can lead to the emergence of resistance to the biocidal product and/or other antimicrobials, including antibiotics. This resistance is usually low, at a level of a few times the initial MIC, but in some cases, it can lead to clinical resistance that has dire implications for human health. Thus, clinical practices where the biocidal product is applied in smaller quantities than recommended (meaning the product will get diluted when applied) or is likely to remain present at low concentration (compared to the recommended concentration for disinfection) or is applied during a too short period of time should be avoided. These recommendations are also valid for the use of biocidal products in household, agricultural or production settings.

There are also reports of stock solutions of chlorhexidine getting contaminated by bacteria and leading to health problems and even death in patients. In some cases, the stock solution was contaminated during production, before it reached the hospital while in others the stock solution or container got contaminated through multiple use, thus contributing to the emergence of resistance.

Further research in the clinical practice (and in other fields) may highlight specific uses of biocidal products that potentiate the development of antimicrobial resistance.

What are the mechanisms that lead to the development of antimicrobial resistance, following the use of biocidal products?

As explained in the report, microorganisms use different strategies to resist biocidal products; they may inactivate the product or modify its target, prevent its entry into the cell or increase its removal from inside the cells. The same mechanisms are used to resist antibacterials and antibiotics; as a result, developing resistance against biocidal products can drive resistance to antibiotics. For instance, bacteria with mutated *fabI* have emerged with high level triclosan resistance (this is an example of modifying the target of the antimicrobial, a strategy that is usually mostly used to resist antibiotics). In addition, bacteria may also express ROS-detoxifying enzymes such as superoxide dismutases (SOD), glutathione peroxidases and peroxiredoxins that may confer low-level resistance to disinfection by hydrogen peroxide or other oxidative disinfectant (this is an example of inactivating the antimicrobial or its toxic by-products); as a result, they may become more tolerant towards antibiotics that kill in part by causing an oxidative stress. Modifying the permeability of the cell envelope through mutations in the LPS pathway or by modifying the expression of porins (this is an example of limiting the entry of antimicrobials inside the cell) is another example of resistance mechanism that is common to the fight against biocides and antibiotics. Finally, the formation of biofilms is

another strategy used by bacteria and fungi that is efficient to limit the entry of biocidal products and antibiotics. This mechanism of resistance is a major issue, as the data analyzed in this report indicate that all biocidal products reviewed here may be subject to increased resistance when cells are embedded in a biofilm matrix. Both these mechanisms may also prevent the entry of other antimicrobial, such as antibiotics.

Another prevalent resistant mechanism seems to be the expression of efflux pumps. These pumps utilize energy to drive the transport of molecules, including antimicrobials, from the inside of the cells to the outside environment and thus prevent the accumulation of toxic molecules inside the cell. They may be already present in the cell and expressed upon exposure to a biocidal product, or they may be shared through horizontal gene transfer. This mechanism of resistance is problematic since efflux pumps that are expressed or shared following the use of biocidal products may also drive the export of other antimicrobials, such as antibiotics that are substrates for efflux pumps, leading to potential health hazard.

Gaps in knowledge and research needs

While a significant amount of research is available on the subject of resistance of microorganisms to biocidal products, there are areas that are severely lacking. In this section we identify the gaps of knowledge and research needs to have a better, fuller understanding of how and how often resistance to biocidal products occur, which microorganisms are affected and whether this resistance is clinically relevant:

- Standardized testing protocols are needed for assessing resistance to biocidal products and cross-resistance to antibiotics, both in test tubes/petri dishes and situations that mimic the uses in practice.

- Standardized testing of commercial biocidal products and research on how different formulation of active substances influence the killing of microorganisms and the development of resistance and cross resistances. Data is scarce, but there is evidence reported in this review that suggests that additional components in the formulation of biocidal products may reduce the risk of emergence of resistance.

- No readily available threshold to establish whether bacterial strains are clinically resistant to biocidal products, which makes it hard to evaluate the risks associated with the use of one biocidal product, even when data on increased resistance is available.

- Majority of data on the emergence of resistance following the use of biocidal products available is about Gram-positive and Gram-negative bacteria. It is unknown whether the general lack of data on yeasts and molds and other microorganisms is because these microorganisms do not readily develop resistance following the use of biocidal product or whether there is a lack of research on the subject.

- The majority of bacteria may be present in biofilms, and this review highlighted the fact that microorganisms in biofilm are much more resistant to biocidal products. There is a lack of data on how this resistance occurs in biofilms, and whether the biocidal products may be used to kill microorganisms in biofilms. Furthermore, current data suggests that biocidal products may either promote or decrease biofilm formation. Additional studies are required to understand the conditions in which biocidal products may promote or decrease biofilm formation.

- To identify the potential risk for biocidal products resistance and cross-resistance, both now and in the future, we need detailed knowledge on the quantity of biocides produced, used and recovered in the environment.

- Comprehensive studies are needed to assess the mechanisms of resistance, the genetic/phenotypic factors involved and the contribution of resistance, tolerance and persistence to the survival of the microorganisms. How biocidal substances influence horizontal gene transfer and how this may increase the spread of AMR determinants should be studied as well.

Recommendations

- Surveillance programs should be developed on a national/European level to monitor resistance and cross-resistance of microorganisms in all areas of biocide usage, in particular the health care setting, veterinary setting, household setting and food industry.

- Communication programs targeting the general public and health sector workers should be developed to increase awareness of resistance and cross resistance related to the use of biocidal products. For example, these could be in the form of a reminder to use a specific quantity of product for a specific duration, as is already done for hands cleaning.

- Good Practices surrounding the use of biocidal substances, especially those that carry a high risk of development of resistance and cross-resistance, should be established in concertation with the health sector and if possible, the manufacturer of the biocidal product. These Good Practices should at least ensure that 1) the in-practice concentration reaches the appropriate level *i.e.,* a sufficient amount is applied on a sufficiently small surface, 2) the appropriate contact time between the biocidal substance and the microorganisms to be decontaminated is respected, 3) after decontamination, the potentially remaining microorganisms are not exposed to sub-lethal levels of the biocidal product for extended periods. For non-volatile products, this may be achieved through rinsing off/wiping.

- There should be adequate protocols for the use of stock solutions of biocidal products and containers of biocidal product to ensure sterility over time.

- Commercially available bioindicators used to assess the efficiency of disinfection are sometimes more susceptible to the action of biocidal products than clinically relevant strains. Bioindicators should ideally not be more susceptible than clinically relevant strains.

- There should be incentives to include the CAS number of chemicals in scientific studies (at least in clinical studies), as this would facilitate interconnections between academic researcher and legislators. The unique formula identification (the UFI) code should also be included to identify biocidal substances in commercial products even if its composition changes.

- The use of biocidal products that carry a high risk for the development of antimicrobial resistance, such as chlorhexidine and QACs, in household products and over-the-counter medication should be reevaluated.

- The use of biocidal products that carry a high risk for the development of antimicrobial resistance, such as chlorhexidine and QACs, should be restricted to applications where these biocides are clearly more adapted or efficient than biocidal products that carry a lower risk for the development of resistance. See note below on the use of hand-rubs and antimicrobial soaps as an example.

A relevant example is the use of hand rubs and antimicrobial soaps. Some commercially available alcohol-based hand rubs contain additional biocidal substances such as chlorhexidine digluconate, triclosan or benzalkonium chloride. A review found that formulations containing additional biocidal substances had no superior efficacy after 3h under the surgical glove when used for the recommended application time (315). Since the data presented in this report clearly indicates a potential risk for the development of resistance and cross-resistance to antibiotics when using biocidal substances such as chlorhexidine, benzalkonium chloride and triclosan, it should be recommended that alcohol-based hand rubs do not contain any additional biocidal substance, except if a clear efficacy improvement can be demonstrated.

Similarly, antimicrobial soaps can be based on different biocidal agents such as chlorhexidine digluconate, povidone iodine, triclosan, benzalkonium chloride, polihexanide or sodium hypochlorite. They are used in health care (surgical hand scrubbing included) and occasionally in the domestic setting (20). Except if a superior efficacy can be proven, antimicrobial soaps containing substances that are likely to lead to the development of resistance should not be used (typically soaps containing chlorhexidine, triclosan or benzalkonium chloride). Antimicrobial soaps containing biocidal substances that are less likely to lead to resistance should be used instead (such as povidone iodine, polyhexanide or sodium hypochlorite). Alternatively, hand scrubbing with regular soap, followed by disinfection of the clean hands with alcohol-based hand rubs (with no additional biocidal substances) may be an option to be considered (20).

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