

ANTISEPTICS: THEIR CHARACTERISTICS, APPLICATION AND CHALLENGES IN THE 21ST CENTURY RESULTING FROM THE SPREAD OF ANTIMICROBIAL RESISTANCE (AMR)

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Abstract: In the context of escalating microbial resistance to antibiotics, antiseptics are gaining prominence as a critical component of infection prevention. Concurrently, the widespread and increasing use of these biocides, particularly within healthcare settings, has prompted concerns regarding their potential contribution to the emergence of reduced microbial susceptibility to them and the phenomenon of cross-resistance to antibiotics. This review focuses on four widely utilized antiseptics: chlorhexidine, octenidine, povidone-iodine, and alcohols. It discusses their antimicrobial activity, mechanisms of action, and applications, including available preparations and the minimum effective concentrations required for reliable pathogen eradication. Current evidence regarding the mechanisms underlying decreased susceptibility to these agents is summarized. Furthermore, the review presents data from studies investigating the impact of prolonged exposure to subinhibitory concentrations of antiseptics on the induction of reduced antimicrobial efficacy and the potential for co-selection of antibiotic resistance. Furthermore, the review presents methods of adaptation of bacteria and fungi to increasing concentrations of antiseptics, including techniques using liquid media - gradient method and incremental method, as well as methods based on solid media. Findings from recent studies suggest that long-term exposure of microorganisms to subinhibitory concentrations of antiseptics may result in reduced effectiveness of these agents and selection of mutants with changed sensitivity to antibiotics.

1. Introduction. 2. Main groups of antiseptics - characteristics, mechanism of action and resistance, available products on the market. 2.1. Chlorhexidine. 2.2. Octenidine. 2.3. Iodophores 2.4. Alcohols. 3. Adaptation to antiseptics. 3.1. Exposure to chlorhexidine and changes in susceptibility profiles. 3.2. Exposure to octenidine and changes in susceptibility profiles. 3.3. Exposure to alcohol / PVP-I and changes in susceptibility profiles. 4. Conclusion.

Keywords: antibiotic cross-resistance, antiseptic adaptation, antiseptic resistance, disinfectants, efflux pumps;

1. Introduction

Antiseptics and disinfectants play a crucial role in preventing infections caused by various pathogens, limiting the spread of multidrug-resistant microorganisms, and maintaining high standards of public and personal hygiene. These biocidal agents are widely used in many areas, including medical, veterinary, industrial, public facilities, and domestic areas settings (Campana and Baffone 2017, Tyski *et al.* 2022, Tyski *et al.* 2024). Increasing attention is being paid to their application in public health settings such as hospitals,

hospices, and long-term care facilities, especially in light of the growing global challenge of antimicrobial resistance (AMR).

Antiseptics are substances that, when applied to the skin, mucous membranes (including the oral cavity), or superficial wounds, are capable of destroying living microorganisms (USP-NF1072). Depending on the intended purpose of use, they may have a prophylactic or therapeutic function. Prophylactic applications include preventing infections through skin disinfection before surgical procedures, hand hygiene in hospitals, or patient bathing prior to medical interventions.

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Therapeutically, antiseptics are used in the treatment of existing infections, such as infected wounds, where they are applied topically and regularly over an extended period. These agents contain antimicrobial substances that can be classified into several key groups, including alcohols, aldehydes, oxidizing compounds (such as hydrogen peroxide, sodium hypochlorite, peracetic acid, ozone, and iodophors), phenolic compounds, and cationic surfactants, which include quaternary ammonium compounds, biguanides, and bipyridines (Łukomska-Szymańska *et al.* 2017). Unlike antiseptics, disinfectants are chemical agents that destroy microorganisms when applied to an inanimate surface. Both antiseptics and disinfectants are critical components of infection prevention strategies in medical facilities, where they are routinely used to reduce infection risk and prevent healthcare-associated infections (HAIs). In recent years, due to the SARS-CoV-2 pandemic and the associated risk of serious health consequences with infection, people's awareness of the threats related to microorganisms has increased. Furthermore, the COVID-19 pandemic has contributed to a significant global increase in disinfectant use. Antisepsis and disinfection have become one of the most important methods of preventing infections, covering homes, hospitals and public spaces. Hand disinfection has become a daily habit, also in public spaces. An increasing number of people have begun to use antiseptics in their homes and workplaces (Babalska *et al.* 2021). Alcohol-based products became the most commonly used antiseptics, while chlorine-based products were the preferred choice for surface disinfection in households (Guo *et al.* 2021). Antiseptics and disinfectants, played a particularly crucial role in the hospital environment, as evidenced by the significant increase in their use in 2020 compared to 2019, before the outbreak of the COVID-19 pandemic. For example, their use increased by 368% in adult wards and by 299% in pediatric wards in 2020 compared to 2019 (Denisiewicz and Denisiewicz 2021). Effective surface disinfection against SARS-CoV-2 includes agents such as ethanol, hydrogen peroxide, sodium hypochlorite, phenols, chlorine-releasing agents, formaldehyde, glutaraldehyde, iodine-releasing compounds, and quaternary ammonium compounds. The WHO particularly recommends phenols, hydrogen peroxide, sodium hypochlorite, ethanol, and ammonium compounds for this purpose (Guo *et al.* 2021).

However, insufficient knowledge among the general public and sometimes even among healthcare personnel regarding the proper use of antiseptics and disinfectants can result in reduced antimicrobial efficacy or the development of microbial resistance. Common issues include improper storage and incorrect usage (e.g., inappropriate concentrations, unsuitable surfac-

es, against inappropriate bioburden, or targeting microorganisms outside the agent's spectrum) (Dindarloo *et al.* 2020). Such misuse may promote the emergence of bacterial strains with reduced susceptibility to biocides. Moreover, the widespread and prolonged use of these agents has been associated with increases in both minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC). This trend was observed following the introduction of chlorhexidine and octenidine into clinical practice. For instance, a comparison of *Staphylococcus aureus* isolates before and after the introduction of these agents revealed increased MIC and MBC values (Hardy *et al.* 2018). A similar pattern was seen in *Enterococcus faecium*, where strains isolated between 1998 and 2015 demonstrated greater tolerance to isopropanol, suggesting that prolonged exposure to alcohol-based antiseptics may have contributed to this adaptation (Pidot *et al.* 2018).

These findings raise concerns about the potential for increased antiseptic tolerance to influence bacterial cross-resistance to antibiotics. It has been observed that long-term exposure to biocides can result in the emergence of mutants with reduced antibiotic susceptibility (Garratt *et al.* 2021). It is worth noting that, before market authorization, the effectiveness of new biocidal products should be evaluated in accordance with European Standards (ENs) (Tyski *et al.* 2022). For antiseptics intended for medical use tests in accordance with the European Pharmacopoeia monograph (2024) are required, which we described in our previous publication (Tyski *et al.* 2022). It should be realized that the above-mentioned effectiveness tests are definitely different from determining activity by the MIC and MBC values.

The aim of this review is to draw attention to the status of currently available antiseptics and products containing them, available on Polish market, and to the possibility of acquiring tolerance or reducing the susceptibility of bacteria, including hospital strains, to antiseptics used in healthcare facilities. Furthermore, we explore the existing knowledge on how bacterial exposure to antiseptics may affect antibiotic resistance profiles, along with the molecular mechanisms underlying these changes.

1. Main groups of antiseptics - characteristics, mechanism of action and resistance, available products on the market

1.1. Chlorhexidine

Chlorhexidine (CHX) belongs to the bisbiguanide, a class of cationic antimicrobial agents. Its structure consists of two symmetrically arranged 4-chlorophe-

nol rings and two guanidine groups connected by a hexamethylene chain (Thangavelu *et al.* 2020). CHX can be obtained in a number of different forms, including acetate, dihydrochloride salts, and digluconate. However, chlorhexidine digluconate (CXG) is the most commonly used due to its high solubility. It exhibits a wide range of efficacy against both Gram-positive and Gram-negative bacteria, although higher CXG concentrations are required in order to combat Gram-negative bacteria. A 4% solution of CXG demonstrates bactericidal activity within just 5 minutes of contact against both Gram-positive and Gram-negative bacteria (Ekizoglu *et al.* 2016). It also effectively eliminates fungi, yeast, dermatophytes, and certain lipophilic viruses. However, its sporicidal properties is only achieved at elevated temperatures (Łukomska-Szymańska *et al.* 2017).

Chlorhexidine at low concentrations exhibits bacteriostatic activity, while at high concentrations it demonstrates bactericidal effects. Its mechanism of action is based on direct interaction with the bacterial cytoplasmic membrane. As a cationic surfactant, CHX binds to the negatively charged cell surface, disrupting the organization of the outer phospholipid layer. It displaces stabilizing divalent cations, leading to decreased membrane fluidity and the formation of hydrophilic domains in its structure. At higher concentrations, increased membrane permeability is observed, resulting in leakage of cytoplasmic contents and ultimately denaturation and precipitation of proteins and nucleic acids (Cieplik *et al.* 2019). This molecular mechanism of action correlates with observed morphological changes induced by chlorhexidine in bacterial cells. Studies have demonstrated that its action leads to the deformation and degradation of the cell wall in both Gram-negative bacteria, such as *Escherichia coli*, and Gram-positive bacteria, such as *Bacillus subtilis*. Upon exposure to chlorhexidine, characteristic indentations were observed on the bacterial cell surfaces, particularly in the tip or cap region of *B. subtilis* and along the trunk of *E. coli* cells, as revealed by scanning electron microscopy (Cheung *et al.* 2012). Furthermore, the number of these indentations increased proportionally with CHX concentration. Transmission electron microscopy (TEM) also showed the formation of “ghost cells” following prolonged CHX exposure (Cheung *et al.* 2012).

Chlorhexidine has also been evaluated for its efficacy against microbial biofilms, which are often less susceptible to antimicrobial substances than planktonic cells. Kean *et al.* (2018) studied the impact of CXG on the biofilm of *Candida* spp., including *Candida auris*. Currently, *C. auris* strains are the most multidrug-resistant pathogenic yeast causing healthcare-associated infections. It has been shown that CHX at a concen-

tration of 0.05% showed high efficacy against planktonic *C. auris* cells, but yeast biofilms, especially mature ones, showed tolerance to such CHX solutions. Increasing the CHX concentration to 2% resulted in complete destruction of early-stage biofilms, as well as a reduction of mature ones. The antiseptic efficacy of 2% chlorhexidine was also tested on interspecies biofilms. The analysis showed that this agent effectively reduced the number of viable cells in single-species biofilms, including *C. auris* NCPF 8973, *S. aureus* NCTC 10,833 and *Staphylococcus epidermidis* RP62A (ATCC 35984). Similar efficacy was observed in dual-species biofilms (*C. auris* with *S. aureus*, and *C. auris* with *S. epidermidis*), where the reduction in cell numbers exceeded $4 \log_{10}$ (Gülmez *et al.* 2022). The effectiveness of CHX against the biofilm of Gram-negative bacteria, i.e. *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, has also been demonstrated. Depending on the strain, the ability of CHX to inhibit biofilm formation and reduce mature biofilm was observed (Hubner *et al.* 2010a; Machuca *et al.* 2019; Perez-Palacios *et al.* 2022).

Antiseptic products with chlorhexidine have been widely used for a long time. Therefore, studies on the basic antimicrobial efficacy of CHX according to EN phase 1 are rarely published. Based on tests conducted in a miniaturized assay according to EN 1040, after 5 minutes of exposure *P. aeruginosa* to different concentrations of CXG, it was shown the highest efficacy at concentrations of 4% and 0.12%, where the bacterial cells reduction was $5.34 \log_{10}$ for both concentrations. For *K. pneumoniae*, a 4% CXG solution achieved $\geq 5 \log_{10}$ reduction, while efficacy dropped below $5 \log_{10}$ at 0.12% and 0.06%. *E. coli* showed the greatest sensitivity to CXG, with a $\log_{10}$ reduction of 5.69 at 4% concentration, but less than $5 \log_{10}$ at lower concentrations (Hornschuh *et al.* 2021).

Unlike phase 1 EN, phase 2 tests are dedicated to a specific area of product application. In the medical area, in phase 2, step 1 of EN 13727 is used to test antibacterial activity, and in phase 2, step 2, several standards are recommended (Tyski *et al.* 2022). In scientific research, modifications are introduced to the methodology according to EN and studies are conducted on wider panels of strains. It has been shown that changes in the chlorhexidine efficacy depending on the presence of isopropyl alcohol. In a study conducted using the quantitative suspension test (EN 13624), a chlorhexidine-based skin antiseptic [2% (w/v) CXG in 70% (v/v) isopropyl alcohol (IPA)] was found to meet the full fungicidal requirements, achieving $> 4 \log_{10}$ cells reductions for *Candida albicans* and *C. auris* in both clean and dirty conditions after 2 minutes of a contact time. In contrast, hand and body wash antiseptic [4%

CXG (v/v)] showed limited efficacy against *C. auris*, achieving reductions in the range of 1.55–2.63 $\log_{10}$ after 2 minutes of exposure in clean conditions and 1.15–2.45 $\log_{10}$ in dirty conditions. For *C. albicans*, the effect was more pronounced, with reductions of 2.83 $\log_{10}$ in clean conditions and 2.78 $\log_{10}$ in dirty conditions after 1 minute, which increased to 3.57 $\log_{10}$ and 3.36 $\log_{10}$, respectively, after 2 minutes. Still, the 4% chlorhexidine gluconate (v/v) met the EN 13624 for hygienic hand washing, requiring a $\geq 2 \log_{10}$ reduction in 1 minute in dirty conditions (Moore *et al.* 2017).

CXG-based impregnated antiseptic wash-mitts [100 g contains 2% CXG and 0.04% benzalkonium chloride] were tested at concentrations of 10%, 50%, 80%, and 97% to evaluate their antifungal efficacy. However, quantitative suspension tests performed according to the EN 13624 demonstrated that none of the concentrations achieved $\geq 4 \log_{10}$ reduction in *C. albicans* ATCC 10231 or two *C. auris* strains (DSM 21092 and DSM 105986) after a 30-second contact time (Gugsch *et al.* 2024).

In the conducted study, following the EN 13727 and EN 13624, the bactericidal and fungicidal efficacy of a 2% CXG solution was evaluated. The results demonstrated that the efficacy of the preparation increased with prolonged exposure time. After 1 minute of contact with *P. aeruginosa* ATCC 15442, a reduction lower than 5 $\log_{10}$ was observed. However, after 5 minutes of exposure, the cells reduction exceeded 5.38 $\log_{10}$. In the case of *E. coli* NCTC 10538, after 1 minute of exposure, the reduction was above 5.52 $\log_{10}$ in clean conditions, while in dirty conditions, it was below 5 $\log_{10}$. After 5 minutes, regardless of the conditions, the reduction exceeded 5.52 $\log_{10}$, indicating full efficacy of the preparation after a longer exposure time. In contrast, *C. albicans* ATCC 10231 displayed a lower sensitivity to 2% CXG, achieving a reduction of 3.52 $\log_{10}$ in clean conditions and 3.27 $\log_{10}$ in dirty conditions after 1 minute. After 5 minutes of exposure, the cells reduction exceeded 4.52 $\log_{10}$, thereby meeting the fungicidal standard (reduction $\geq 4 \log_{10}$). In the case of *Aspergillus brasiliensis*, no fungicidal activity was observed, as the reduction remained below the required threshold in both clean and dirty conditions after 1 and 5 minutes of exposure (Şahiner *et al.* 2019).

Studies on the efficacy of CHX against SARS-CoV-2 have yielded inconsistent findings. Some laboratory investigations report that CHX-containing mouthwashes are ineffective at inactivating the viruses, (Komine *et al.* 2021) while others show that they can reduce viral load temporarily but not permanently. A study comparing 0.05% CHX with 0.05% cetylpyridinium chloride demonstrated a modest but statistically

significant decrease in viral load among SARS-CoV-2-positive patients. Interestingly, a similar reduction was observed in patients using placebo irrigation (0.9% NaCl), suggesting that this reduction may be primarily due to the effect of mechanical irrigation (Bonn *et al.* 2023). Another study demonstrated that a 0.12% CHX mouthwash temporarily suppressed the SARS-CoV-2 viral load in saliva, reducing it to undetectable levels for up to two hours. However, after four hours, the viral load increased again, indicating a short duration of this effect (Yoon *et al.* 2020). Although some studies suggest limited effectiveness of chlorhexidine in reducing viral load, other research has shown that CHX mouthwashes and throat sprays can offer a promising method for eliminating SARS-CoV-2 from the throat in COVID-19 patients. The combination of a 0.12% CHX mouthwash and throat spray demonstrated the highest efficacy, with 86.0% of patients achieving viral clearance from the throat, compared to 62.1% in the group using mouthwash alone. This was significantly higher than the 5.5% of patients in the control group using only mouthwash and 6.3% in the control group using both mouthwash and spray (Huang and Huang 2021).

Chlorhexidine, may result in various adverse effects. Commonly reported side effects include contact skin irritation and taste disturbance. In rare cases, allergic reactions such as occupational asthma, skin rash, photodermatitis or anaphylaxis may occur. Prolonged use may also lead to tooth and tongue staining (Łukomska-Szymańska *et al.* 2017). Surfaces covered with plaque tend to exhibit more intense staining and a greater extent of calculus formation compared to those that are plaque-free. This suggests that performing an initial professional teeth cleaning before the use of CHX can help mitigate its undesirable side effects, especially with long-term use (Zanatta *et al.* 2010).

CHX is widely used as an active ingredient in various products, acting as an antiseptic either alone or in combination with other substances. A summary of commercially available products, including their concentrations and indications, is presented in Table I. It is extensively utilized in dentistry as a mouthwash to aid in the management of gingivitis during dental interventions and to reduce plaque accumulation. There are additional mouthcare gels with strengths of 1% and 0.2%, toothpastes with 0.05% CHX, and biodegradable “chips” of CXG that can be put into periodontal pockets in conjunction with subgingival debridement (Brookes *et al.* 2020). Higher concentrations of CHX (0.2%) demonstrate better plaque-inhibiting effects compared to lower concentrations (0.12% and 0.06%). CHX at a 0.2% concentration is an effective agent used

as a mouth rinse, demonstrating efficacy in reducing *Streptococcus mutans* and *Lactobacillus*. However, they are associated with a higher risk of adverse effects, such as loss of taste and numbness (Haydari *et al.* 2017). Consequently, increasing attention has been directed

toward natural alternatives, such as cocoa bean husk and ginger-based rinses, which have shown potential in reducing *S. mutans* and *Lactobacillus* counts with a lower risk of adverse effects.

Table I
Selected antiseptic products available on Polish market, and their indications

Commercially available products	Active ingredients	Concentration	Product type	Indications
Products with chlorhexidine as a main ingredient				
Aseptall	chlorhexidini digluconatis	0.12%	oral spray	for gum inflammations, post-dental procedures, canker sores, chapped corners of the mouth
ChloraPrep	chlorhexidini digluconatis, 2-propanolum	2% v/v 70% v/v	skin antiseptic applicator	for skin disinfection before surgical procedures
Chlorhexidin puder	chlorhexidini digluconatis	1%	powder	for care and protection of skin areas exposed to infection, supporting the regeneration of irritated or damaged skin
Curaprox Perio Plus+ Focus	chlorhexidini digluconatis	0.5%	toothpaste	helps maintain gum health and regenerates them, prevents tartar formation, eliminates dental plaque, for local use
Curasept ADS DNA 205	chlorhexidini digluconatis	0.05%	mouthwash	especially recommended for people wearing orthodontic appliances or implants, it inhibits the development of dental plaque
Decontaman Pre Wipes	chlorhexidini digluconatis	2%	body wash wipes	for skin disinfection before surgical procedures
ELGYDIUM Perioblock PRO	chlorhexidini digluconatis	0.12%	toothpaste	for irritated, sensitive, or bleeding gums and dental plaque
Eludril Classic	chlorhexidini digluconatis, alcohol	0.1% 43% v/v	mouthwash	for adjunctive treatment for periodontics and implantology, for patients with prosthetic restorations or implants
Eludril Extra	chlorhexidini digluconatis	0.2%	mouthwash	for individuals with sensitive oral mucosa, for irritated and bleeding gums, before and after dental procedures, supplementary use during dental treatment
Elugel	chlorhexidini digluconatis	0.2%	dental gel	for patients wearing orthodontic braces, supplementary use after periodontic procedures, implant and surgical procedures
GUM Butler ParoeX	chlorhexidini digluconatis	0.06%	toothpaste	for use with implants, dentures, orthodontic appliances, protects delicate gums, reduces gum inflammation, helps prevent plaque build-up, provides long-term protection against gum disease
Gum Paroex	chlorhexidini digluconatis	0.12%	mouthwash	for reduction of dental plaque accumulation, relief of sensitive gums, maintenance of gum tissue health
Hydrex S	chlorhexidini digluconatis	4%	solution	for washing hands, for disinfecting the skin of hands and skin before surgery

Manusan	chlorhexidini digluconatis	4%	solution	for hygienic and surgical hand washing, body and hair
MEDISEPT Velodes Soft	chlorhexidini digluconatis, 2-propanolum	(0.5g + 60g)/100g	solution	for hygienic and surgical hand disinfection
OrthoKIN Mint	chlorhexidini digluconatis	0.06%	mouthwash	for people wearing orthodontic braces
Spirytusu Hibitanowego 0,5% ATS	chlorhexidini digluconatis	0.5%	solution	for disinfecting the hands of medical personnel before and after contact with patients, for disinfecting the skin of patients before injections and surgical procedures, for disinfecting the surgical field
Spitaderm	chlorhexidini digluconatis, 2-propanolum, hydrogenii peroxidum 30 per centum	(70g + 0.5g + 1.5g)/100g	solution	for hygienic and surgical hand disinfection before punctures, surgeries, injections
Products with octenidine as a main ingredient				
Octaseptal	octenidinum dihydrochloridum, phenoxyethanol	(0.1g + 2g)/100g	aerosol	for antiseptic treatment of not very extensive wounds and disinfection of the skin, mucous membranes, oral cavity, in the treatment of minor burn and ulcerative wounds, in children (including for the care of the umbilical stump)
Octeangin	octenidinum dihydrochloridum	2.6 mg/tab.	lozenges	for use in short-term adjuvant treatment of inflammation of the oral cavity and throat mucosa
Octeniderm	octenidinum dihydrochloridum, 1-propanolum, 2-propanolum	(0.1g + 30g + 45g)/100g	solution	for skin disinfection before surgical procedures
Octenisept	octenidinum dihydrochloridum, phenoxyethanol	(0.1g + 2g)/100g	solution	for disinfection and supportive treatment of small, superficial wounds and pre-procedural skin disinfection for non-surgical procedures.
Septisse	octenidinum dihydrochloridum, phenoxyethanol	(0.1g + 2g)/100g	aerosol	for skin disinfection before surgical procedures, care of the umbilical stump, postoperative sutures, disinfection of the oral cavity
Products with iodophors as a main ingredient				
Betadine	povidonum iodinum	10%	ointment	for local treatment of burns, wounds, abrasions, trophic ulcers, skin infections
Betadine	povidonum iodinum	75 mg/ml	solution	for washing hands before surgery and hygienic disinfection of hands
Braunoderm	povidonum iodinum, 2-propanolum	(1g + 50g)/100g	solution	for disinfection of intact skin before surgery, injections, punctures, catheterization
Jodi Gel	povidonum iodinum	10%	gel	for disinfecting wounds and skin before surgical procedures, in stomatitis, in primary and secondary local skin infections
PV Jod 10%	povidonum iodinum	100 mg/g	solution	for disinfecting wounds, especially superficial ones and after surgical procedures, as well as burns, scabs and ulcers, prevention and treatment of infections of the skin and mucous membranes

Products with alcohols as a main ingredient				
Desderman N	ethanolum (96%), 2-biphenylol	(79g + 0,1g)/100g	solution	for hygienic and surgical hand skin disinfection, the preparation is recommended for health service facilities
Kodan Tinktur Forte	1-propanolum, 2-propanolum, 2-biphenylolum	(10g + 45g + 0.2g)/100g	solution	for skin disinfection before surgical procedures, blood collection, wound dressing, for hygienic hand disinfection, prevents skin fungal infections
Mikrozid AF liquid	ethanolum (94%), 1-propanolum	(25g + 35 g)/100g	solution	for disinfection of surfaces of medical devices
Mikrozid AF Wipes JUMBO	ethanolum 96%, 1-propanolum	(25g + 35g)/100g	wipes	for disinfection in medical clinics, hospitals (including neonatal and neonatal wards), public places
Primasept med	1-propanolum 2-propanolum, 2-biphenylolum	(10g + 8g + 2g)/100g	solution	for disinfecting and washing hands and body
Promanum pure	ethanolum, 2-propanolum	(78.1g + 10g)/100g	solution	for hygienic and surgical disinfection of hands with sensitive skin
Sensivia	ethanolum, 2-propanolum, acidum lacticum	(45g + 28g + 0,3g)/100g	solution	for hygienic and surgical disinfection of hand skin
Septoderm	ethanolum, 2-propanolum	(45g + 30 g)/100g	gel	for hygienic and surgical hand disinfection
Sirafan Speed	1-propanolum, 2-propanolum	(25g + 35g)/100g	solution	for disinfection of areas in contact with food (tables, slicers)
Skinman Soft	2-propanolum, benzalkonii chloridum, acid undecylenicum	(60g + 0.3g + 0.1g)/100g	solution	for hygienic hand disinfection, for long-term use by people with sensitive skin, for versatile use in medical facilities
Skinsept color	ethanolum, alcohol benzylicus, 2-propanolum	(45.54g + 1g + 27g)/100g	solution	for skin disinfection before surgery, injections, punctures, blood collection and vaccinations
Skinsept Pur	ethanolum (96%), 2-propanolum, alcohol benzylicus	(46g + 27g + 1g)/100g	solution	for skin disinfection before surgeries, injections, punctures, vaccinations, blood collection, dressing changes.
Softa-man	ethanolum 96%, 1-propanolum	(47.9g + 18g)/100g	solution	for hygienic and surgical hand disinfection
Softasept N uncolored	ethanolum 96%, 2-propanolum	(78.83g + 10g)/100g	solution	for skin disinfection before surgical procedures, before venous injections and punctures
Sterillium	1-propanolum, 2-propanolum	(45g + 30g)/100g	solution	for hand skin disinfection

Daily bathing with CHX has been proven effective in preventing infections, especially in the hospital setting. The use of CHX baths in intensive care unit reduces the risk of healthcare-associated infections (HAI), in particular central line-associated bloodstream infections (CLABSI) and infections caused by methicillin-resistant *S. aureus* (MRSA) (Frost *et al.* 2016). Regularly bathing patients with 2% CHX-impregnated washcloths can lower bloodstream infection rates in hospitals by 30% compared to non-CHX methods

(Climo *et al.* 2013). Higher CHX concentrations, such as 4%, have shown even greater efficacy. One study observed a 40.4% reduction in HAIs when patients were bathed with 4% CHX followed by rinsing with water (Pallotto *et al.* 2019).

It is well known that bacteria acquire resistance or develop tolerance to biocides. Efflux pumps are one of the key mechanisms by which bacteria acquire resistance to antiseptics. Genes encoding efflux pumps

can be located in chromosomes as well as in mobile element such as plasmids, integrons, and transposons. The six main classes of efflux pumps are the major facilitator (MFS) superfamily, the ATP-binding cassette (ABC) superfamily, the resistance-nodulation-division (RND) superfamily, the small multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) superfamily and the proteobacterial antimicrobial compound efflux (PACE) superfamily (Kuznetsova *et al.* 2025). Efflux pumps that actively re-

move disinfectants from bacterial cells to the outside are summarized in Table II. The MFS family includes chlorhexidine extruded pumps such as QacA, QacB and SmvA. The ABC family consists of transporters like AdeABC. The RND family includes pumps such as AcrAB-TolC, EfrAB, MexAB-OprM, MexCD-OprJ, MexXY, and SdeAB. The SMR family primarily contains the KpnEF and Smr pumps. The MATE family includes the MepA pump, while the PACE family features the AceI transporter involved in CHX extrusion.

Table II
Bacteria efflux pumps extruded antiseptics

Family of efflux pump	Efflux pump	Species	Gene location	Antiseptic	References
MFS	EmrAB	<i>S. enterica</i>	chromosome	triclosan	(Rensch <i>et al.</i> 2014)
	LmrS	<i>S. aureus</i>	chromosome	benzalkonium chloride	(Kernberger-Fischer <i>et al.</i> 2018)
	MdeA	<i>S. aureus</i>	chromosome	benzalkonium chloride	(Huang <i>et al.</i> 2004)
	MdrL	<i>L. monocytogenes</i>	chromosome	benzethonium chloride	(Romanova <i>et al.</i> 2006)
	NorA	<i>S. aureus</i> , <i>S. epidermidis</i>	chromosome	benzalkonium chloride, cetrimide, acriflavine	(Furi <i>et al.</i> 2013; Qingzhong <i>et al.</i> 2015; Costa <i>et al.</i> 2018)
	NorB	<i>S. aureus</i>	chromosome	cetrimide	(Qingzhong <i>et al.</i> 2015)
	QacA	<i>S. aureus</i>	plasmid	chlorhexidine, benzethonium chloride	(Noguchi <i>et al.</i> 1999)
	QacB	<i>S. aureus</i>	plasmid	chlorhexidine	(Furi <i>et al.</i> 2013)
	SmvA	<i>P. aeruginosa</i> , <i>K. pneumoniae</i>	chromosome	chlorhexidine, octenidine	(Wand <i>et al.</i> 2019; Bock <i>et al.</i> 2021)
ABC	EfrAB	<i>E. faecalis</i> , <i>E. faecium</i>	chromosome	chlorhexidine, triclosan	(Lerma <i>et al.</i> 2014)
	PatAB	<i>S. pneumoniae</i> , <i>S.pseudopneumoniae</i>	chromosome	acriflavine	(Robertson <i>et al.</i> 2005; Alvarado <i>et al.</i> 2017)
RND	AdeABC	<i>A. baumannii</i>	chromosome	chlorhexidine, octenidine, benzalkonium chloride	(Meyer <i>et al.</i> 2022)
	AcrAB-TolC	<i>S. enterica</i> , <i>E. coli</i> , <i>K. pneumoniae</i>	chromosome	chlorhexidine, triclosan	(Mcmurry <i>et al.</i> 1998; Webber <i>et al.</i> 2008; Curiao <i>et al.</i> 2015)
	AcrEF	<i>S. enterica</i>	chromosome	triclosan	(Rensch <i>et al.</i> 2014)
	MexAB-OprM	<i>P. aeruginosa</i>	chromosome	chlorhexidine ¹ , triclosan	(Schweizer 1998; Hashe-mi <i>et al.</i> 2019)
	MexCD-OprJ	<i>P. aeruginosa</i>	chromosome	chlorhexidine, benzalkonium chloride, triclosan	(Chuanchuen <i>et al.</i> 2001; Morita <i>et al.</i> 2003)
	MexEF-OprN	<i>P. aeruginosa</i>	chromosome	triclosan	(Chuanchuen <i>et al.</i> 2001)
	MexXY	<i>P. aeruginosa</i>	chromosome	chlorhexidine ²	(Tag ElDein <i>et al.</i> 2021)
	OqxAB	<i>E. coli</i>	plasmid	benzalkonium chloride, triclosan	(Hansen <i>et al.</i> 2007)
	SdeAB	<i>S. marcescens</i>	chromosome	chlorhexidine, benzalkonium chloride	(Maseda <i>et al.</i> 2009)
	SmeDEF	<i>S. maltophilia</i>	chromosome	triclosan	(Hernández <i>et al.</i> 2011)
	TriABC- OpmH	<i>P. aeruginosa</i>	chromosome	triclosan	(Fabre <i>et al.</i> 2021)

SMR	EmrE	<i>E. coli</i>	plasmid	benzalkonium chloride, acriflavine	(Nishino and Yamaguchi 2001)
	KpnEF	<i>K. pneumoniae</i>	chromosome	chlorhexidine, triclosan, benzalkonium chloride	(Srinivasan and Rajamohan 2013)
	QacG	<i>Staphylococcus</i> spp.	plasmid	benzalkonium chloride,	(Heir <i>et al.</i> 1999)
	QacH	<i>S. saprophyticus</i>	plasmid	benzalkonium chloride	(Heir <i>et al.</i> 1998)
	QacJ	<i>S. aureus</i> , <i>S. simulans</i> , <i>S. intermedius</i>	plasmid	benzalkonium chloride	(Bjorland <i>et al.</i> 2003)
	QacZ	<i>E. faecalis</i>	plasmid	benzalkonium chloride	(Braga <i>et al.</i> 2010)
	Smr	<i>S. aureus</i>	plasmid	chlorhexidine, benzalkonium chloride	(Noguchi <i>et al.</i> 1999)
MATE	AbeM	<i>A. baumannii</i>	chromosome	triclosan, acriflavine	(Su <i>et al.</i> 2005)
	MepA	<i>S. aureus</i>	chromosome	chlorhexidine, benzalkonium chloride, cetrimide	(Costa <i>et al.</i> 2013)
PACE	AceI	<i>A. baumannii</i>	chromosome	chlorhexidine	(Hassan <i>et al.</i> 2015)

¹ proteomic analysis of mutants obtained after exposure to chlorhexidine, showed increased expression of the MexA protein, a component of the MexAB-OprM pump, ²increased expression of the *mexX* gene

Efflux pump Smr from SMR family and MepA pump from MATE family play an main role in the mechanisms of *S. aureus* resistance to antiseptic, including CHX (Noguchi *et al.* 1999; Costa *et al.* 2013). Additionally, *qacA* and *qacC* genes, which are located in plasmids, have been shown to increase CHX resistance in *S. aureus*. Moreover, exposure to benzalkonium chloride can induce *qacC* expression, thereby enhancing CHX tolerance (LaBreck *et al.* 2020).

In a study analyzing 1050 *S. epidermidis* isolates, 63 exhibited reduced sensitivity to CHX (MIC $\geq$ 4 μ g/ml) (Addetia *et al.* 2019). Among these, 9 isolates carried the *qacA* gene, while *qacB* was absent. In addition, the *smr* gene was present in 51 isolates. Notably, a novel *qacA* allele was identified, encoding a modified QacA protein with two amino acid substitutions. This new allele, designated *qacA4*, was located in plasmid pAQZ1 and found in the highly resistant and pathogenic ST2 clone. The *qacA4* gene has been shown to play an important role in increasing CHX resistance, as loss of this gene resulted in a 4-fold reduction in the CHX MIC values, from 4 μ g/ml to 1 μ g/ml.

Exposure to 4% CHX through daily bathing has been linked to increased CHX tolerance in MRSA isolates. Using the modified broth microdilution method in line with the Clinical and Laboratory Standards Institute (CLSI) guidelines, isolates from CHX-exposed patients showed MIC values ranging from 1 to 8 μ g/ml, with MIC $\geq$ 4 μ g/ml occurring three times more fre-

quently than among strains isolated from unexposed patients. Further, the possibility of transferring *qac* genes was analyzed. It was shown that long-term exposure to CHX predisposes to the acquisition of *qacA/B* genes, depending on the clone. This phenomenon was particularly pronounced in the case of clone ST22, in which the frequency of *qacA/B* genes in CHX-exposed strains was significantly higher compared to the unexposed group. A similar trend was also observed in the case of clone ST45, but the increase in the frequency of gene occurrence was less pronounced (Htun *et al.* 2019). Transferring the *qacA* gene between bacteria *via* plasmids is one potential method for the spread of CHX resistance. The *qacA* gene was discovered to be transferable from a CHX-resistant MRSA strain to an *E. coli* strain that had previously been CHX-sensitive. Transfer increased the CHX MIC values in *E. coli* from $\leq$ 0.25 to $\geq$ 16 μ g/ml. Genetic studies confirmed the existence of the *qacA* gene in the recipient strain, indicating that CHX resistance genes can be transferred between bacterial species (Bes *et al.* 2021). Unlike Gram-positive, Gram-negative bacteria extrude CHX mainly *via* the RND family of efflux pumps, including the AcrAB-TolC (*E. coli*, *K. pneumoniae*, *Salmonella enterica* subsp. *enterica*), MexCD-OprJ (*P. aeruginosa*), SdeAB (*Serratia marcescens*), AdeABC (*A. baumannii*) systems (Table II). In addition, the occurrence of the biocide resistance genes (BRGs) such as *cepA*, *qacE Δ 1* and *qacE*, which encode efflux pumps, has been de-

scribed in Gram-negative bacteria (Zhang *et al.* 2019). These genes are transferred by plasmids and transposons. It has been shown that the CepA pump is associated with *K. pneumoniae* resistance to CHX (Fang *et al.* 2002).

The expression of efflux genes is dependent on local as well as global regulators. Recently, it has been shown that exposure to biocides can cause mutations in these regulatory genes or in the regions surrounding efflux pump genes. In *K. pneumoniae*, a single DNA mutation was found in the intergenic region between *smvR* and *smvA* after exposure to CHX. SmvA pump extruded two main antiseptic CHX and OCT. The SmvR protein plays a regulatory role and inhibits the expression of *smvA*. This mutation probably interferes with the mechanism of this regulation, weakening inhibition and leading to increased *smvA* expression. Exposure to antiseptics also leads to the accumulation of multiple mutations in different locations of the bacterial genome. Additionally, E359K substitutions or deletions were detected in the *malT2* gene, which encodes an HTH-type transcriptional activator regulating the maltose operon. However, exposure of *Enterobacter cloacae* to CHX caused mutations in the *bamE* gene, which is an assembly factor for outer membrane proteins, and in the *betI* gene, which is a member of the TetR/AcrR family of transcriptional regulators (Lescat *et al.* 2021). The overproduction of the AcrAB-TolC efflux pump in *Enterobacteriales* depends on both local and global regulators. Among the global regulators, increased overproduction of MarA, SoxR, and RamA has been associated with the overexpression of *acrAB-TolC* operon contributing to CHX resistance (Curiao *et al.* 2015).

1.2. Octenidine

Octenidine dihydrochloride (OCT) is a positively charged surfactant belonging to the bispyridine group. Its structure contains two independent cationic active centers connected by a long aliphatic hydrocarbon chain. OCT demonstrates a wide antimicrobial spectrum, being effective against Gram-positive cocci, including MRSA strains, and Gram-negative bacteria. It also targets plaque-forming bacteria, including *Actinomyces* and *Streptococcus* spp., as well as *Chlamydia*, *Mycoplasma*, and various fungi (Hubner *et al.* 2010b). It shows limited virucidal effectiveness against hepatitis B and herpes simplex viruses (Sathiyamurthy *et al.* 2016). Recently, the OCT-based formulation was found to be effective against SARS-CoV-2 virus (Smeets *et al.* 2021, Steinhauer 2022 *et al.* 2022). Moreover, OCT is a potentially active against *Acanthamoeba* trophozoites

and cysts at concentrations used in commercially available products (Hamad 2023, Wekerle *et al.* 2020).

Studies on OCT's mechanism of action have shown that first point of its attachment in Gram-negative bacterial cells is the outer membrane. As a cationic molecule, OCT has a high affinity for anionic bacterial surface components, e.g. lipopolysaccharides. Due to electrostatic interactions, OCT binds to the surface of *E. coli* cells and then penetrates through the lipopolysaccharide (LPS) layer into the interior of the outer membrane. Analysis of zeta potential changes in response to increasing OCT concentrations revealed that neutralization of the negative surface charge of cells occurs already at a very low concentration of octenidine (10^{-6} %). At this stage, no inhibition of bacterial growth was observed, which indicates that surface neutralization is the first step of action, but not sufficient to kill the cell. The hydrocarbon chains of OCT rapidly interact with the hydrophobic core of the outer membrane, leading to its significant disruption through the so-called hydrophobic mismatch. As a result, subsequent OCT molecules can penetrate deep into the bacterial cell, reaching the inner membrane. To confirm that OCT also interacts with the inner membrane, a depolarization assay with the membrane potential-sensitive dye, i.e. 3,3'-dipropylthiadicarbocyanine iodide (DiS-C₃-5), was used. Upon disruption of the membrane integrity, the dye is released, which causes an increase in the fluorescence signal. Application of 0.0001% OCT induced a rapid increase in fluorescence, indicating that OCT effectively depolarizes the inner membrane. As a consequence, the integrity of both the outer and inner membranes is disrupted by OCT, leading to cell lysis (Malanovic *et al.* 2020).

Ponnachan *et al.* (2019) investigated the effect of OCT on yeast cells. They showed that OCT affects *C. auris* cell integrity in a concentration-dependent manner, leading to its damage and, at higher concentrations, full disintegration. Electron microscopy studies showed that after 6 hours of incubation with 1 µg/ml octenidine resulted in a reduction of the cell envelope of *C. auris* (clinical isolates), which suggests the beginning of cell disintegration. As the octenidine concentration increased to 2 µg/ml and 5 µg/ml, the cell structure was increasingly damaged. More serious damage to yeast cells, leading to leakage of their contents, was visible after 24 hours of incubation at a concentration of 2 µg/ml, and complete lysis occurred at a concentration of 5 µg/ml. OCT also exhibits antifungal activity against *C. albicans* (Fang *et al.* 2023). At 1 µM, a cells reduction of 3.22 log₁₀ was observed, while 2 µM caused a 5.32 log₁₀ reduction. At 4 µM, OCT completely eliminated *C. albicans* cells and inhibited bio-

film formation by 92.54%, but mature biofilms were eradicated by 71.88%. At 8 μM , mature biofilms were completely removed.

In the case of *S. aureus*, 1 mM OCT reduced planktonic cells by $> 3 \log_{10}$ and 2 mM OCT led to complete eradication. It also inhibited biofilm formation and removed mature biofilms at 5 mM and 10 mM concentrations (Amalaradjou and Venkitanarayanan 2014). OCT was also effective against *P. aeruginosa* biofilms after 30 minutes of exposure to a 0.1% solution (Junka *et al.* 2014). *A. baumannii* biofilm was completely eliminated by OCT after 5–10 minutes of exposure to a 0.9% (15 mM) and 0.6% (10 mM) solutions, respectively (Narayanan *et al.* 2016).

OCT effectiveness tests were also carried out in accordance with EN standards including additional pathogenic species. In the medical area, ENs require testing only on *C. albicans* (effectiveness against yeast), and on *S. aureus*, *P. aeruginosa* and *E. coli* (effectiveness against bacteria). The fungicidal assay by quantitative suspension tests were performed according to EN 13624 (a phase 1 step 1), including MDR yeast *C. auris* (Gugsch *et al.* 2024). The yeast-killing efficacy of OCT-impregnated washing mitts was demonstrated at concentrations of 80%, 50% and 10% against three *Candida* species tested (*C. auris* DSM 21092, *C. auris* DSM 105986 and *C. albicans* ATCC 10231) after a 30-second contact time under low organic load conditions. At lower concentrations, *C. albicans* showed greater resistance compared to *C. auris*. At 1% concentration, *C. auris* strains achieved $> 4 \log_{10}$ reductions, with *C. albicans* showing 2.19 $\log_{10}$ reductions. An OCT concentration of 0.5% proved to be ineffective against both *C. auris* strains (Gugsch *et al.* 2024).

In a study conducted in accordance with EN 13727, a bactericidal effect of OCT was demonstrated. Exposure to a 0.01% OCT solution led to a $> 5 \log_{10}$ reduction in clinically relevant bacterial species, including *E. coli*, *K. pneumoniae*, *E. cloacae*, *A. baumannii*, and *P. aeruginosa*, within just 1 minute of contact in both clean and dirty conditions. In addition, a concentration of 0.0001% OCT required a longer exposure time of 2.5 minutes to achieve a reduction of $> 5 \log_{10}$ (Alvarez-Marín *et al.* 2017).

The antiseptic preparation containing 0.1% OCT and 2% phenoxyethanol showed significant antimicrobial efficacy in the test conducted using the quantitative suspension method based on EN 13727. After 30 minutes of exposure, a 4.77 $\log_{10}$ reduction in *P. aeruginosa* DSM-939 was observed with 0.3 ml of the solution, as well as a 6.18 $\log_{10}$ reduction in both *S. aureus* DSM-799 and the clinical MRSA strain. Additionally, when the tested volume of the antiseptic solution was

increased to 1 ml, complete elimination of MRSA and *P. aeruginosa* biofilm was observed within 72 hours (Rembe *et al.* 2020). The study conducted according to EN 13727 showed that the application of a solution containing 0.1% OCT and 2% phenoxyethanol (Octenisept) to wound exudate reduced the number of microorganisms by at least 5 $\log_{10}$ in just 15 seconds. Meanwhile, a wound irrigation solution with 0.05% OCT (Octenilin) also demonstrated antimicrobial efficacy but required 30 seconds of contact to achieve a similar level of bacterial reduction (Augustin *et al.* 2023).

OCT is commonly available as a 0.1% solution or aerosol, usually in combination with 2% phenoxyethanol, e.g. on the product Octenisept. It's used for skin disinfection before surgical procedures, as well as for managing wounds, mucous membranes, and conditions in the oral cavity. The antimicrobial efficacy of Octenisept is the result of the activity of both ingredients. OCT can also be formulated with 1-propanol or 2-propanol. Lozenges containing 2.6 mg of OCT are used in the treatment of inflammatory conditions of the oral cavity. A summary of commercially available products, including their concentrations and indications, is presented in Table I. In the case of chronic wounds, it is recommended to use products containing 0.05% OCT, which are widely available in the form of gels or rinsing solutions, often enriched with a surfactant such as ethylhexylglycerin. The gel formulation is especially useful for antiseptic treatment in burn victims, exhibiting greater efficiency than silver and iodophores in these instances. A solution of 0.1% OCT and 2% phenoxyethanol is efficacious for the management of acute, contaminated, and traumatic wounds, including those colonized by MRSA (Kramer *et al.* 2018). The alcohol-based skin disinfectant containing OCT (propan-1-ol 30%, propan-2-ol 45%, octenidine dihydrochloride 0.1%) demonstrates greater effectiveness in reducing and preventing microbial recolonization around the insertion sites of central venous catheters and extracorporeal catheters compared to the disinfectant containing propan-2-ol (63%) and benzalkonium chloride (Lutz *et al.* 2016). OCT is an important component of strategies to prevent hospital infections and improve patient safety in intensive care units (ICUs). The use of 0.08% OCT impregnated wipes for patient bathing has been shown to be effective in the prevention of primary bacteremia associated with ICU stay (Schaumburg *et al.* 2024).

OCT-based antiseptics are used for a much shorter period of time than CHX-based antiseptics. Therefore, the molecular basis for the decrease in bacterial and fungal susceptibility to OCT is less well understood.

Recently, such analyses have been conducted on strains from the *Enterobacterales* order. Studies have shown that *K. pneumoniae* can adapt to increased exposure to OCT by mutations in the SmvA pump (A363V, L364Q, Y391N, A363T, A368T, A474V) belonging to the MFS family (Wand *et al.* 2019). The AdeABC efflux pump of the RND family has been reported to extrude OCT from the bacterial cell in *A. baumannii* (Meyer *et al.* 2022). Efflux pumps that actively remove disinfectants from bacterial cells to the outside are summarized in Table II. A single nucleotide deletion in *K. pneumoniae* was also found in the genes encoding the RamR protein, which belongs to the TetR/AcrR family of transcriptional regulators. In *Klebsiella oxytoca*, mutations were identified in the gene encoding the Bm3R1 protein, which also belongs to the TetR/AcrR family and in *E. cloacae* in the gene encoding OmpX, a precursor of the outer membrane protein (Lescat *et al.* 2021).

1.3. Iodophores

Iodophors are compounds that release iodine, created by combining iodine with a solubilizing agent in water-based solutions, as iodine itself is unstable in water. One common example of an iodophor is povidone-iodine (PVP-I). Povidone-iodine is created by combining iodine molecules with polyvinylpyrrolidone (PVP), which makes it water-soluble. In this compound, PVP functions as a carrier for iodine, allowing it to absorb and transport iodine without chemically reacting with it. The iodine itself is the active ingredient in PVP-I (Babalska *et al.* 2021). PVP facilitates the release of free iodine near the cell membranes of microorganisms, which then penetrates the membrane, causing its damage and loss of structural integrity. After entering the cell, iodine denaturates the structure of nucleic acids and disrupts the basic energy processes of the cell, such as electron transport, cellular respiration, and protein synthesis. These cell function disorders ultimately lead to cell death (Williamson *et al.* 2017). The more diluted the PVP-I solution, the higher the concentration of free iodine in it. This occurs because dilution weakens the binding of iodine to its carrier. Consequently, solutions with lower concentrations (around 0.1–1%) tend to act faster and are more effective at killing bacteria compared to those with higher concentrations, such as the 10% solution (Babalska *et al.* 2021). Determination of the efficacy of PVP-I at different pH according to EN 27027 and the National Committee for Clinical Laboratory Standards M27-A2 showed that with increasing pH, the antibacterial efficacy of 10% PVP-I was significantly reduced against *S. aureus* and *P. aeruginosa* (Wiegand *et al.* 2015). The

presence of organic compounds, such as bovine serum albumin, can also diminish the effectiveness of PVP-I as a disinfectant. Studies have shown that the presence of albumin leads to a reduction in the antibacterial efficacy of PVP-I. Specifically, an albumin concentration of 0.01875% caused a decline in the antibacterial activity by PVP-I (Kapalschinski *et al.* 2017).

PVP-I exhibits broad-spectrum antimicrobial activity, targeting a wide range of microorganisms, including both Gram-positive and Gram-negative bacteria, *Mycobacterium*, fungi (i.e. *Candida* and *Trichophyton* species), and protozoa. With prolonged exposure, it also demonstrates activity against spores and various viruses, such as multiple strains of the Influenza virus and Ebola virus (Lachapelle *et al.* 2013; Williamson *et al.* 2017).

In the case of a variety of clinical *Candida* spp. isolates, including *C. albicans* associated with vulvovaginal candidiasis, PVP-I at an 8% concentration demonstrated significant fungicidal activity in a test conducted according to EN 1275 (phase 1). After 60 minutes of exposure, 8% PVP-I eliminated all *Candida* spp. isolates, achieving a reduction of $\geq 4 \log_{10}$ (Hacıoglu *et al.* 2022). Furthermore, a quantitative suspension test by EN 13624 (phase 2, step 1) was used to evaluate yeasticidal activity, showing that 10% PVP-I demonstrated very high efficacy against *C. auris*, reducing the yeast count to $> 4.5 \log_{10}$ within 2 minutes of contact. However, when tested against *C. albicans* ATCC 10231, this time was not sufficient (Moore *et al.* 2017). Şahiner *et al.* (2019) also evaluated the bactericidal and fungicidal efficacy of 7.5% PVP-I solution according to EN 13727 and EN 13624. *P. aeruginosa* ATCC 15442 and *E. coli* K12 NCTC 10538 showed high sensitivity, reaching a bacterial cells reduction of more than $5 \log_{10}$ after 1 minute of exposure, regardless of conditions. In contrast, *S. aureus* ATCC 6538 required 5 minutes to reach the same reduction level in dirty conditions, while in clean conditions, effectiveness was observed after 1 minute. *C. albicans* ATCC 10231 showed a similar trend: the required $4 \log_{10}$ reduction was reached after 5 minutes of exposure under dirty conditions, and after 1 minute under clean settings. Further, no fungicidal activity was observed against *A. brasiliensis* in either clean or dirty conditions, as the log reduction remained below the required threshold after 1 and 5 minutes of exposure.

PVP-I is available in various formulations, including antiseptic ointments, solutions, and gels, most commonly in 7.5% and 10% concentrations. These preparations are used for the treatment of burns and wounds, as well as for preoperative skin disinfection and surgical hand scrubbing. A summary of commer-

cially available products, including their concentrations and indications, is presented in Table I.

Clinical trials have shown that a combination of 1% PVP-I (containing 10% free iodine) and 50% isopropyl alcohol is as effective as 2% CXG in 70% ethanol in preventing surgical site infections following cardiac and abdominal surgeries (Widmer *et al.* 2024). Similarly, no significant difference was observed in infection risk reduction between 5% PVP-I in 69% ethanol and 2% CHX in 70% isopropanol for cardiac surgery patients (Boisson *et al.* 2024).

PVP-I-based mouth rinses are considered a valuable protective tool against infections in the oral cavity and respiratory tract. Tests conducted according to the bactericidal quantitative suspension test EN 13727 demonstrated that a 0.7% povidone-iodine solution, diluted to 0.23% (1:30 dilution), exhibited significant bactericidal activity against *K. pneumoniae* DSM 16609 and *Streptococcus pneumoniae* ATCC 49619, achieving a cells reduction of over 5 log₁₀ within just 15 seconds of exposure. Compared to plain soft soap, the scalp and skin cleanser containing 7.5% PVP-I is proven to be more effective in eliminating *E. coli* and mouse norovirus (MNV) (Eggers *et al.* 2018b).

PVP-I-based antiseptic products are also effective in preventing and eradicating microbial biofilms. Research has demonstrated that *C. auris* biofilms exhibit increased tolerance to PVP-I as compared to planktonic cells. PVP-I concentrations in the range of 1.25–2.5% were required to inhibit the biofilms growth after 5 min of exposure. Prolonged exposure to 10–30 minutes reduced required concentrations to 0.625–1.25%. The highest efficacy in eliminating biofilms was demonstrated by a 10% PVP-I, which completely destroyed all stages of the biofilm (Kean *et al.* 2018). Also, a 10% PVP-I solution demonstrated high efficacy in eliminating *S. aureus* biofilm, achieving a 99% reduction after 30 minutes of exposure (Guimarães *et al.* 2012). The efficacy of PVP-I in eliminating MSSA and MRSA biofilm from titanium surfaces was assessed. Irrigation for 3 minutes with a PVP-I solution at a concentration of 0.8% for MSSA and 1.6% for MRSA resulted in a ≥ 99.9% reduction of biofilm (Semeshchenko *et al.* 2025). Overnight incubation with subinhibitory concentrations of PVP-I (0.17%, 0.35%, 0.7%) suppressed the ability of *S. epidermidis* 1457 and *S. aureus* RN4220 to form biofilms. In *S. epidermidis*, this inhibition was due to an increase in the level of *icaR*, a transcriptional repressor of the *icaADBC* operon, which is responsible for the production of polysaccharide intercellular adhesin (PIA). In *S. aureus*, no correlation was found between reduced *icaADBC* operon and *icaR* gene expression (Oduwole *et al.* 2010).

A 10% PVP-I effectively reduced the number of viable cells in both single-species biofilms (*C. auris* NCPF 8973, *S. aureus* NCTC 10,833, *S. epidermidis* ATCC 35984) and multi-species biofilms (*C. auris* + *S. aureus*, and *C. auris* + *S. epidermidis*), reducing their numbers by more than 4 log₁₀. The presence of *Staphylococcus* spp. in mixed biofilms did not improve the ability of *C. auris* to persist under PVP-I exposure, indicating its high efficacy against multi-species biofilms (Gülmez *et al.* 2022). On the other hand, 7.5% PVP-I did not demonstrate full efficacy in eradicating *P. aeruginosa* biofilm (Junka *et al.* 2014). After 15 minutes of exposure, a 15% reduction in biofilm was noted, while after 30 minutes the efficacy increased to 66%.

The activity of PVP-I against viruses is extremely important. PVP-I can be employed as a nasal spray or nasal irrigation for the nasopharyngeal clearance of the SARS-CoV-2 virus in patients with COVID-19. Among various concentrations, a 0.5% solution used for nasal irrigation has shown the greatest effectiveness, while among nasal sprays, the best results were observed with the 0.6% solution (Arefin *et al.* 2022). PVP-I demonstrates excellent virucidal activity against the Ebola virus. PVP-I formulations, including 4% skin cleanser, 7.5% surgical scrub, 10% PVP-I solution, and 3.2% PVP-I in 78% alcohol, significantly decreased EBOV virus titers, achieving a cells reduction ranging from 5.66 to 6.84 log₁₀ after 15 seconds of application (Eggers *et al.* 2015). Furthermore, inactivation tests conducted according to the virucidal quantitative suspension test EN 14476 demonstrated that 0.23% PVP-I solution effectively inactivated SARS-CoV, MERS-CoV and influenza A virus (H1N1) (Eggers *et al.* 2018a).

1.4. Alcohols

Among alcohols, ethanol and isopropanol (propan-2-ol, 2-propanol) are most commonly used as antiseptics. They are effective against Gram-positive and Gram-negative bacteria, *Mycobacterium*, yeasts, and molds (Williamson *et al.* 2017; Stauf *et al.* 2019). Ethanol is capable of inactivating all enveloped viruses, including Coronaviridae, Herpes, Vaccinia, and Influenza viruses, as well as several non-enveloped viruses such as Adenovirus and Rotavirus. In contrast, isopropyl alcohol is ineffective against non-enveloped viruses like Adenovirus but remains effective against lipid-enveloped viruses, including coronaviruses (Parikh and Parikh 2021). However, neither ethanol nor isopropanol eliminate bacterial spores. The optimal bactericidal efficacy is noted during the 60%–90% concentration ranges, with a significant reduction in effectiveness occurring when concentrations fall be-

low 50% (Williamson *et al.* 2017). Alcohols exert their antimicrobial effects by denaturing and coagulating proteins, which leads to a loss of structural integrity of cell membranes. This results in increased membrane permeability, which is manifested by leakage of intracellular components. As a result, cellular processes, including metabolic functions and enzyme activity, are impaired. Ultimately, this cascade of events causes cell lysis (Elekhrawy *et al.* 2020).

The antimicrobial efficacy of alcohol depends on the specific conditions under which it is used. The presence of viscosity-increasing substances can hinder alcohol penetration into microbial cells, reducing its disinfectant effectiveness. For example, in mucus samples (both artificial and sputum), the bacterial survival rate exceeded 10% after application of an alcohol-based disinfectant, indicating significantly compromised antibacterial effectiveness. Additionally, ethanol diffusion ability into mucus was inversely related to its viscosity, which was associated with increased bacterial resistance (Hirose *et al.* 2017). Ethanol is widely used in professional disinfection practices in both healthcare and veterinary settings. A summary of commercially available products, including their concentrations and indications, is presented in Table I. In healthcare facilities, a solution of 69% ethanol combined with 5% PVP-I has been shown to be effective for skin antisepsis prior to surgical procedures, for example cardiac surgery (Boisson *et al.* 2024). In veterinary medicine, for instance, 74.1% ethanol mixed with 10% propan-2-ol is used for skin antisepsis in dogs prior to medical procedures (Eigner *et al.* 2023). No instances of alcohol tolerance have been observed in bacteria like staphylococci and streptococci, nor have any mechanisms of acquired alcohol resistance been discovered (Williamson *et al.* 2017).

Hand sanitizer gel and foam containing 70% ethanol demonstrated high antimicrobial efficacy in *in vitro* time-kill tests according to ASTM E2783-10. At 15 seconds of contact, *S. marcescens* reduction was $> 5.8 \log_{10}$ (gel) and $> 4.7 \log_{10}$ (foam), and MRSA reduction was $> 5.8 \log_{10}$ (gel) and $> 4.2 \log_{10}$ (foam). ASTM E1174 testing has confirmed the effectiveness of these products. After the first application, a reduction of at least $2 \log_{10}$ in microorganism count was observed, and after the tenth application, the reduction reached at least $3 \log_{10}$, for both 5 ml and 2 ml volumes (Edmonds *et al.* 2012).

Bactericidal activity against enterococci *Enterococcus hirae* ATCC 10541, *E. faecium* ATCC 6057 and *Enterococcus faecalis* ATCC 47077 was assessed in accordance with EN 13727. After 5 min exposure to 40% ethanol significant differences in species tolerance were

observed. *E. faecium* and *E. faecalis* showed the lowest susceptibility, with reductions of only 1.24 and 4.11 $\log_{10}$, respectively. On the other hand, *E. hirae* showed the highest sensitivity at 40% concentration, with cells reduction of 7.31 $\log_{10}$. Ethanol concentrations of 50% or higher consistently resulted in reductions of at least 5 $\log_{10}$ after just 30 seconds of exposure (Suchomel *et al.* 2019).

The fungicidal activity of ethanol was tested in a quantitative suspension test, according to EN 13624. Reference strains were included: *C. albicans* ATCC 10231, *Candida tropicalis* ATCC 13803, *A. brasiliensis* ATCC 16404, and *Aspergillus niger* ATCC 6275, as well as clinical antifungal-resistant isolates. After 1 minute of exposure, ethanol at 50% concentration showed efficacy against yeasts, achieving $\geq 4.0 \log_{10}$ reduction, while an 80% concentration was effective against molds (Stauf *et al.* 2019).

The effect of alcohol solutions on biofilm formation depends on the bacterial species and alcohol concentration. In one study, a comparison of 41 ethanol concentrations from 0% to 20% revealed that low concentrations stimulated *S. aureus* biofilm formation, with the highest biofilm stimulation noted at 7% ethanol. Biofilm formation then gradually decreased with increasing ethanol concentration up to 20%. Furthermore, extending incubation from 24 to 48 hours increased biofilm production (Vaezi *et al.* 2020). Importantly, higher concentrations of ethanol, starting from 30% and upwards, reduce the ability to form biofilms. Alonso *et al.* (2018) showed that therapy with both concentrations of 40% and 70% ethanol almost 100% reduced metabolic activity in 72-hour biofilms of *S. aureus* ATCC 29213, *S. epidermidis* (clinical isolate), *E. faecalis* ATCC 33186, *C. albicans* ATCC 14058, and *E. coli* ATCC 25922. However, 70% ethanol was more effective against 48-hour biofilms.

Similarly, exposure to subinhibitory concentrations of ethanol (1/4 MIC, 2.5% and 1/2 MIC, 5.0%) significantly increased the ability of *Salmonella* Enteritidis to form biofilm, with a stronger effect observed at 5.0%. This suggests that sublethal ethanol stress may trigger mechanisms that promote biofilm development. It was examined whether there were changes in attachment genes (*adrA*, *csgB*, *csgD*), quorum sensing genes (*luxS*, *sdiA*), and sRNAs (*ArcZ*, *CsrB*, *OxyS*, *SroC*). Expression analysis showed that the *luxS* gene was significantly upregulated, with 2.49-fold and 10.08-fold increases at 2.5% and 5% ethanol, respectively. The remaining genetic elements examined did not alter their activity in response to ethanol exposure. Similarly, in the case of *P. aeruginosa*, ethanol at concentrations of 1% and 2% increased biofilm formation (He *et al.* 2022).

In tests conducted according to EN 13727 and EN 13624, isopropanol at a concentration of 70% has been shown to have an effective bactericidal and fungicidal effect, regardless of the presence of organic substances. The preparation provided a reduction of $> 5 \log_{10}$ for bacteria (*S. aureus* ATCC 6538, *E. coli* K12 NCTC 10538, *P. aeruginosa* ATCC 15442 and *E. hirae* ATCC 10541) and $> 4 \log_{10}$ for fungi (*C. albicans* ATCC 10231 and *A. brasiliensis* ATCC 16404) after 1 and 5 minutes of exposure, in both clean and dirty conditions (Şahiner *et al.* 2019).

2. Adaptation to antiseptics

To investigate how bacteria adapt to increasing concentrations of antiseptics, methods involving a series of passages in a concentration gradient are used. There are two the most commonly used approaches to perform stepwise transfers of microorganisms in liquid media: (a) subsequent transfers of the obtained mutants to new media with a whole series of antiseptic dilutions in 96-well microtiter plates - gradient method, (b) step-by-step transfer of each obtained mutants to a new medium with a 1.5-2 times higher concentration of the antiseptic in the tube - increment method (Krajewska *et al.* 2024). In both methods, sub-MIC concentrations of antiseptics are also included in the tests. In the gradient method, a 96-well microtiter plate was prepared as for determining the MIC value of the tested compound as antiseptic. Such a subsequent transfer approach to the study of the ability to adapt to chlorhexidine has been described for a individual bacterial / yeast clinical isolates and laboratory strains (Zheng *et al.* 2022) and for mix oral microorganisms present in supragingival plaque samples (Fruh *et al.* 2022). To perform the next passage, the bacterial inoculum is taken from the highest concentration of antiseptic at which growth still occurs (the sub-MIC value) and transferred to series of fresh medium containing antiseptic dilutions. Following incubation, the MIC was redetermined and another passage was performed in the same manner (Fruh *et al.* 2022). An example of such a procedure is the approach used by Zheng *et al.* (2022) in which the cultured overnight of *P. aeruginosa* were transferred to LB broth containing various CHX concentrations ($1/2 \times \text{MIC}$, $1 \times \text{MIC}$, $2 \times \text{MIC}$, and $4 \times \text{MIC}$). After 24 h, the bacterial culture suspension that showed visible growth at the highest CHX concentration were transferred to series of fresh medium containing antiseptic dilutions and resubjected to the same procedure. This method resulted in *P. aeruginosa*

mutants with CHX MICs $\geq 64 \mu\text{g/ml}$ after 10 passages.

Another approach by the increment method was described by Zhang *et al.* (2019) in which clinical *K. pneumoniae* strains were serially passaged in test tubes with gradually increasing concentrations of antimicrobial agents. Bacteria were first inoculated into tube containing 10 ml of nutrient broth supplemented with the initial concentration of antiseptic ($1/2 \text{ MIC}$ of chlorhexidine). The cultures were incubated at 35°C for up to 48 h. Then, 100 μl of the bacterial suspension from the tube was transferred to a tube containing twice increased concentration of antiseptic (e.g., the chlorhexidine concentration increased with each passage) and incubated. Bacterial cultures were further passaged until the maximum level of tolerance was reached, which corresponded to an MIC of $128 \mu\text{g/ml}$. However, Gregorchuk *et al.* (2021) used a modified increment method. An overnight culture of *E. coli* K-12 was inoculated into liquid LB medium containing $1/5$ of the MIC values of chlorhexidine. The following day, the resulting culture was re-inoculated into fresh liquid LB medium also containing $1/5$ of the MIC of CHX and grown until 12 days to expose the culture to prolonged sub-inhibitory CHX. The obtained culture was then inoculated into medium at a concentration equal to the MIC value of CXH and, in the next step, above the CHX MIC value. Yet another modification of the increment method was proposed by Karpiński *et al.* (2025) who studied in a 96-well plate the ability of *P. aeruginosa* strains to adapt to antiseptics - CHX and OCT - in the range of 0.5% to 4.5% concentrations in which they are used in commercial antiseptic products.

In contrast to the previous methods, another approach used by Bleriot *et al.* (2020) consisted of *K. pneumoniae* exposed for two weeks to $1/4 \text{ MIC}$ of CHX in liquid media with aeration. The antiseptic was replaced every 24 h. In this case, the CHX concentration was kept constant and bacteria were exposed to this sub-MIC concentration of the antiseptic throughout the experiment.

Another method of long-term exposure of bacteria to OCT was used in a study using a hospital sink drain system that was connected to an automated drain model. The procedure included a 21-day acclimatization period, during which the system functioned without the addition of antiseptic, allowing the original microbiota to be maintained. Then, for 62 days, water flow was started four times a day for 40 seconds, and after 10 seconds a preparation containing 0.3% OCT added 10 seconds after the start of each flow cycle. After this period, the antiseptic was discontinued for 35 days and

subsequently resumed for an additional 21 days (Garrett *et al.* 2021).

Unlike liquid media, solid media can also be useful for examining the impact of bacterial exposure to antiseptics, including changes in bacteria's sensitivity to antiseptics and on the bacterial resistance profiles to drugs. A Soft Agar Gradient Evolution (SAGE) Plates method in which a concentration gradient is created by the diffusion of an antiseptic agent in agar can be used (Krajewska *et al.* 2024). In this approach, a concentration of antiseptic equivalent to half the minimum inhibitory concentration is added to molten nutrient agar and poured onto a petri dish set at an angle, creating a sloped layer. After the agar had solidified, the dish was placed horizontally and another layer of nutrient agar was poured on top, this time without the addition of antiseptic. Thanks to the angle setting in the first stage, a concentration gradient was created - in places where the enriched layer was thicker, the diffusion of the biocide was greater, and in thinner places - weaker. Subsequently, bacteria were inoculated onto the prepared plate, starting from the area with the lowest antiseptic concentration. Colonies that grew in the area with the highest antiseptic concentration were subcultured to another plate prepared in the same manner but with twice the antiseptic concentration. The procedure was continued, doubling the concentration of the antiseptic each time, until no growth occurs. This method allowed obtaining *E. coli* mutants with a twofold increase in antiseptic MIC values after hydrogen peroxide exposure (32 to 64 µg/ml) and *P. aeruginosa* mutants after benzalkonium chloride exposure (64 to 128 µg/ml). In contrast, *S. aureus* exposed to CHX showed no change in CHX MIC (remained at 7.8 µg/ml), but developed cross-resistance to oxacillin (the MIC value rising from 0.2 to 2 µg/ml) (Adkin *et al.* 2022).

Another technique for preparing solid medium-based plates with an antiseptic concentration gradient was used by Cowley *et al.* (2015) The aim of this study was to assess the effect of the product formulation on the development of bacterial insensitivity. Substances in the form of an aqueous solution and as a formulation (50 µl) were applied to agar plates with TSA medium using an automated spiral plater, which allows obtaining a 100-fold concentration gradient of substances on the plate. The plates prepared in this way were dried for one hour, and then a pure culture of bacteria was applied to them. Bacteria growing at the highest concentration were cultured on a new plate containing the same concentration gradient. When growth was obtained over the entire concentration

range, the bacteria were inoculated to new plate with a 5-fold higher concentration of the substance. This procedure was repeated 14 times (Cowley *et al.* 2015).

2.1. Exposure to chlorhexidine and changes in susceptibility profiles

CHX is the most extensively studied antiseptic. Both Gram-negative and Gram-positive bacteria, as well as fungi, have been long-term exposed to CHX. CHX has sometimes been used as a reference antiseptic.

Zhang *et al.* (2019) found that prolonged exposure to CHX, using the increment method, increased the resistance of *K. pneumoniae*. In all three strains, the CHX MIC reached 128 µg/ml, and this adaptive resistance remained stable even after about 10 passages in CHX-free medium. Furthermore, the adapted to CHX strains developed cross-resistance to colistin. This CHX resistance was associated with higher expression of the *cepA* gene in all strains, whereas the *qacE* and *qacE1* genes were not found. Additionally, all adapted strains carried mutations in *PmrB*, particularly Leu82Arg. The Leu82Arg mutation is suspected to play a key role in colistin resistance. What's more, those strains had different growth rates than their wild-type counterparts. Similarly, another study has shown acquired cross-resistance to colistin in two CHX-exposed clinical strains of carbapenemase-producing *K. pneumoniae*: ST258-KPC3 and ST846-OXA48. After exposure to chlorhexidine, the MIC of the tested strains increased 4-fold for ST258-KPC3 from 9.8 µg/ml to 39.1 µg/ml and for ST846-OXA48 from 19.5 µg/ml to 78.2 µg/ml. In addition, a 32-fold increase in the MIC of colistin was observed in strain ST846-OXA48. No differences in susceptibility were observed for the other antibiotics tested, as no changes in MIC values were detected. In the ST258-KPC3 strain, the expression of the *smvA* gene, which encodes the efflux pump, was increased (log₂ fold change: 3.635), while ST846-OXA48 was characterized by high expression of the *pmrD* (log₂ fold change: 2.36) and *pmrK* (log₂ fold change: 1.57) genes, which are related to lipid A synthesis. In the plasmid of the ST846-OXA48CA strain, a novel toxin/antitoxin system (PemI/PemK) was identified. It was further observed that expression of gene encoding the PemK toxin resulted in reduced biofilm formation (Bleriot *et al.* 2020). All microbial mutants obtained after exposure to chlorhexidine, changes in their antiseptic sensitivity and drug susceptibility profiles, and genotypic changes are listed in Table III.

Table III
Bacterial and yeast mutants obtained by stepwise exposure to the following antiseptics:
chlorhexidine, octenidine, povidone-iodine, and ethyl alcohol

Microorganism	Antiseptic used for exposure	Changes in sensitivity to (x-fold increase in MIC value) ^a		Phenotypic/Genotypic changes in mutants	References
		Antiseptic	Antibiotics/ Chemotherapeutics		
<i>Citrobacter</i> spp.	octenidine	2-fold increase in MIC	4-fold increase in MIC for ampicillin, piperacillin, ceftazidime, and chloramphenicol, 2- to 4-fold increase in MIC for ciprofloxacin and meropenem	no significant difference in the growth rates and biofilm formation, significant virulence reduction / mutations in <i>marR</i> and <i>envZ</i>	(Garratt <i>et al.</i> 2021)
<i>Enterobacter</i> spp.	octenidine	2-fold increase in MIC	cross-resistance to ciprofloxacin, chloramphenicol, and ceftazidime	growth retardation, no significant difference in biofilm formation, no change in virulence / deletions SNPs ^b in <i>malT</i> and <i>torA</i> , D21E mutation in <i>SmvA</i>	(Garratt <i>et al.</i> 2021)
<i>E. coli</i>	chlorhexidine	2- to 4-fold increase in MIC	no changes in antibiotic susceptibility	cell shape change: narrowing, reduced average cell length, more permeable membranes / changes in protein abundance levels: upregulation: GadE, NfsA, NfsB, MdfA, PmrB, LpxL, downregulation: CadA, Lon; changes in gene expression levels: upregulation: <i>emrAB</i> , <i>ompX</i> , <i>ompA</i> , , <i>gadE</i> , <i>mdtEF</i> , <i>gadABC</i> , <i>cadA</i> , <i>hdeABD</i> , <i>ydeN</i> downregulation: <i>mliA</i> , <i>cdaR</i> , <i>rob</i> , <i>soxS</i> , <i>ompT</i> , <i>ompF</i>	(Gregorchuk <i>et al.</i> 2021)
	ethyl alcohol	no relevant MIC changes	no changes in antibiotic susceptibility	nt ^c / nt	(Shepherd and Parker 2023)
<i>K. pneumoniae</i>	chlorhexidine	16- to 32-fold increase in MIC	128-fold increase in MIC for colistin	different growth capacities / 8.88 to 11.95-fold higher expression of efflux pump gene <i>cepA</i> , mutation Leu82Arg in <i>PmrB</i>	(Zhang <i>et al.</i> 2019)
		4-fold increase in MIC	32-fold increase in MIC for colistin	nt / overexpressed gene <i>smvA</i> , high expression of the <i>pmrD</i> and <i>pmrK</i> , identification of PemI/PemK TA system, PemK toxin expression reduced biofilm formation	(Bleriot <i>et al.</i> 2020)
		4-fold increase in MIC	8-fold increase in MIC for colistin	colonies of irregular shape and rough surfaces / nt	(Hashemi <i>et al.</i> 2019)
<i>A. baumannii</i>	chlorhexidine	4-fold increase in MIC	16-fold increase in MIC for colistin	colonies of irregular shape and rough surfaces / nt	(Hashemi <i>et al.</i> 2019)

<i>P. aeruginosa</i>	chlorhexi- dine	8-fold increase in MIC	32-fold increase in MIC for colistin	colonies of circular shape, slightly rough surface with undulating margins / increased expression of OprF, LptD, TolB, TolA, MurD, PagL, ClpB, SecG, SecB, SecA, ArcA, ArcB, ArcC, MexA, AceE, AceF, FadA, FabV, AcpP1, Pil proteins	(Hashemi <i>et al.</i> 2019)
		4- to 32-fold increase in MIC	decreased susceptibility to imipenem, meropenem, levofloxacin, ciprofloxacin, ceftazidime, cefepime, and tobramycin, cross-resistance to imipenem and ciprofloxacin	nt / upregulation of <i>mexA</i> , <i>mexC</i> , <i>mexE</i> , <i>mexX</i> , downregulation of <i>oprD</i>	(Zheng <i>et al.</i> 2022)
		2- to 22-fold increase in MIC	no changes in antibiotic susceptibility	nt / nt	(Karpiński <i>et al.</i> 2025)
		≥8-fold increase in MIC	2- to 4-fold increase in MIC for amikacin, cefepime, and meropenem , 2-fold increase in MIC for ciprofloxacin, ceftazidime, and colistin	changes in membrane permeability / upregulation of <i>mexX</i>	(Tag ElDein <i>et al.</i> 2021)
	octenidine	16-fold increase in MIC	no changes in antibiotic susceptibility	no significant difference in the growth rates and biofilm formation, no change in virulence / mutations in <i>smvR</i> (TetR regulator)	(Garratt <i>et al.</i> 2021)
		4- to 32-fold increase in MIC	4-fold increase in MIC for gentamicin and colistin, 2-fold increase in MIC for amikacin and tobramycin	all mutants maintained unchanged virulence in the wax moth larvae <i>G. mellonella</i> model, three showed a decreased growth rate / nt	(Shepherd <i>et al.</i> 2018)
		3- to 12-fold increase in MIC	no changes in antibiotic susceptibility	nt / nt	(Karpiński <i>et al.</i> 2025)
	povidone- -iodine	4-fold increase in MIC	no changes in antibiotic susceptibility	nt / nt	(Karpiński <i>et al.</i> 2025)
	ethyl alcohol	no relevant MIC changes	15-fold increase in MIC for imipenem and aztreonam, 10-fold increase in MIC for gentamicin, 8-fold increase in MIC for ceftazidime	reduced growth / nt	(Shepherd and Parker 2023)
	ethyl alcohol	no relevant MIC changes	4-fold increase in MIC for gentamicin	nt / nt	(Shepherd and Parker 2023)
<i>S. aureus</i>	chlorhexi- dine	4- to 8-fold increase in MIC	4- to 512-fold increase in MIC for tetracycline and amikacin, 2- to 512-fold increase in MIC for cefepime and gentamicin, 8- to 512-fold increase in MIC for meropenem, 2- to 64-fold increase in MIC for ciprofloxacin	nt / nt	(Wu <i>et al.</i> 2016)
		2- to 4-fold increase in MIC	no changes in antibiotic susceptibility	nt / mutations in <i>mepA</i> , <i>purr</i> , <i>pldB</i> , <i>glpD</i> , and <i>mprF</i>	(Renzoni and François <i>et al.</i> 2017)
	povidone- -iodine	2-fold increase in MIC	nt	inhibition biofilm formation, reduced hemolytic activity / down-regulation of <i>icaA</i> , <i>icaD</i> , <i>eno</i> , <i>epbs</i> , <i>fib</i> , <i>hla</i>	(Barakat <i>et al.</i> 2022)

<i>S. epidermidis</i>	ethyl alcohol	no relevant MIC changes	no changes in antibiotic susceptibility	nt / nt	(Shepherd and Parker 2023)
<i>S. oralis</i>	chlorhexidine	2-fold increase in MIC	decrease in susceptibility to erythromycin, increased MIC for clindamycin, amoxicillin, ampicillin	no significant difference in biofilm formation / nt	(Früh <i>et al.</i> 2022)
<i>Streptococcus</i> spp.	chlorhexidine	2- to 8-fold increase in MIC	resistance to erythromycin and tetracycline, intermediate resistance to penicillin G and ampicillin, intermediate or resistance to cefuroxime and amoxicillin/clavulanic acid	increased biofilm formation / presence of ARGs: <i>tetM</i> , <i>patA</i> , <i>patB</i> , <i>mefI</i> , <i>pbpX2</i> , <i>int</i> , <i>xis</i>	(Auer <i>et al.</i> 2022)
<i>G. adiacens</i>	chlorhexidine	4-fold increase in MIC	decreased susceptibility to erythromycin, clindamycin, increased MIC for penicillin G, tetracycline, cefuroxime, ciprofloxacin	slight increase in the ability to biofilm formation / nt	(Früh <i>et al.</i> 2022)
<i>C. albicans</i>	octenidine	no relevant MIC changes	no changes in antibiotic susceptibility	nt / nt	(Spettel <i>et al.</i> 2025)
	chlorhexidine	no relevant MIC changes	no changes in antibiotic susceptibility	nt / nt	(Spettel <i>et al.</i> 2025)
<i>N. glabratus</i>	octenidine	2-fold increase in MIC	no changes in antibiotic susceptibility	nt / nt	(Spettel <i>et al.</i> 2025)
	chlorhexidine	4-fold increase in MIC	64- to 256-fold increase in MIC for fluconazole, 4- to 128-fold increase in MIC for posaconazole, 32- to 125 increase in MIC for voriconazole, 8- to 64-fold increase in MIC for itraconazole, 32- to 512-fold increase in MIC for isavuconazole	nt / mutations in <i>PDR1</i> , mutations in <i>PMA1</i> , overexpression of <i>CDR1</i>	(Spettel <i>et al.</i> 2025)

ARG - antibiotic resistance genes, ^a x-fold increase in MIC compared to the parental strain, ^b single nucleotide polymorphism, ^c not tested

Cross-resistance to colistin was found not only in *K. pneumoniae* but also in *A. baumannii* and *P. aeruginosa* strains using the gradient method (Hashemi *et al.* 2019). For *K. pneumoniae* the MIC value of colistin increased from 2 µg/ml to 16 µg/ml, for *A. baumannii* from 1 µg/ml to 16 µg/ml, and for *P. aeruginosa* from 1 µg/ml to 32 µg/ml. A potential mechanism of cross-resistance to colistin may result from LPS modification, which could be suggested by morphological changes in the obtained mutants. Colonies of *A. baumannii* and *K. pneumoniae* strains were characterized by irregular shape and rough surface, while colonies of *P. aeruginosa* had slightly rough structure and wavy edges. Differences in the protein composition of the resistant *P. aeruginosa* strain were detected. These involved increased overproduction of proteins including outer membrane porin F, LPS assembly protein LptD, Tol-Pal system protein TolB, Tol-Pal system protein TolA, UDP-N-acetylmuramoylalanine-d-glutamate ligase, lipid A deacylase PagL, Chaperone protein ClpB, Sec proteins, and efflux pump MexA (Hashemi *et al.* 2019).

Zheng *et al.* (2022) reported that *P. aeruginosa* mutants selected through exposure to CHX by gradient method exhibited 4- to 32-fold higher MICs. These mutants showed reduced susceptibility to multiple antibiotics, including imipenem, meropenem, levofloxacin, ciprofloxacin, ceftazidime, cefepime, and tobramycin. Reduced CHX susceptibility was linked to efflux pump activity. qRT-PCR showed significantly upregulated expression of *mexA*, *mexC*, *mexE*, and *mexX*, and downregulation of *oprD* gene (Zheng *et al.* 2022).

Tag ElDein *et al.* (2021) used two methods to assess the effect of CHX on the selection of *P. aeruginosa* strains exhibiting cross-resistance to antibiotics. Resistant strains were obtained using gradient method and by a single exposure to a lethal concentration of CHX. Of the 28 mutants, 12 showed at least an 8-fold MIC increase of CHX compared to the parent strain. All of these mutants exhibited a 2-fold to 4-fold MIC increase of amikacin. Furthermore, seven of them became resistant to meropenem (MIC change from 4 to 8 or to 16 µg/ml), while six shifted from full susceptibility to intermediate resistance to ciprofloxacin (MIC change from 1 to 2 µg/ml). Three mutants developed intermediate resistance to cefepime (MIC change from 4 to 16 µg/ml). In addition, some strains had higher MICs of amikacin, ceftazidime, and colistin yet remained susceptible to these antibiotics. Two of the obtained mutants demonstrated significantly decreased membrane permeability, whereas the remaining mutants showed increased permeability or no change. Exposure to 0.5 MIC of CHX resulted in an increase in *mexX* gene expression. In 7 out of 12 isolates, this increase was high,

reaching up to a 43-fold change. In contrast, 2 isolates showed no overexpression of *mexX* following CHX exposure.

The study conducted by Gregorchuk *et al.* (2021) showed that *E. coli* after adaptation to increasing concentrations of CHX, using increment method, did not develop cross-resistance to any of the tested antibiotics. On the contrary, it resulted in increased susceptibility to tobramycin, with a reduction in the MIC from 16 to 4 µg/ml. They also showed increased susceptibility to antimicrobials, including QAC, cetyltrimethylammonium bromide, and cetyltrimethylammonium bromide. At the same time, as a result of this adaptation, three isolates showed reduced sensitivity to CHX, with their MIC increasing from 2- to 4-fold compared to the initial MIC value: changes from 2 µg/ml to 4 µg/ml in two mutants, and to 8 µg/ml in one mutant. Proteome analysis of the strain showing the highest phenotypic stability revealed changes in the abundance of many proteins, e.g. porin OmpF, lipid synthesis/transporter MlaA, efflux pump MdfA, proteins controlling acid resistance (GadE, CdaR), and antimicrobial stress-inducible pathways Mar-Sox-Rob. Scanning electron microscopy (SEM) imaging revealed that adaptation to CHX caused a change in cell shape, resulting in narrowing, and in 2/3 of the isolates, it also reduced average cell length. In addition, changes in the cell membrane were investigated using a fluorescent dye (propidium iodide) that does not pass through the membrane. The results demonstrated that strains adapted to CHX had more permeable cell membranes than the wild-type strain. These findings suggest that *E. coli* adaptation to increasing concentrations of CHX results in significant phenotypic changes that may be detected using both visual and fluorescence methods.

Wu *et al.* (2016) investigated whether subinhibitory exposure to the antibiotics, chlorhexidine and *Rhizoma coptidis* extract (RCE) induced cross-resistance or reduced susceptibility in *Staphylococcus* spp. including 14 clinical isolates and the reference strain *S. aureus* ATCC 25923. After exposure to sublethal concentrations of chlorhexidine, most isolates showed no major change in susceptibility, but six isolates showed a 4- to 8-fold increase in MICs, with MIC changes from 1.56-0.78 µg/ml to 6.25 µg/ml, respectively. *S. aureus* ATCC 25923 exhibited cross-resistance to tetracycline and cefepime (MICs changes from ≤ 1 µg/ml to 8 µg/ml). One isolate showed a > 512-fold increase in MIC of amikacin, tetracycline, and gentamicin. No significant change in susceptibility was observed for ciprofloxacin in 4 isolates, gentamicin in 5 isolates, amikacin in 2 isolates, cefepime in 3 isolates, and meropenem in 5 isolates. Additionally, 7 strains exhibited reduced

sensitivity to RCE. Most strains exposed to sub-MIC tetracycline showed a 4-fold increase in MICs, except for one strain. *S. aureus* ATCC 25923 also developed resistance to ciprofloxacin and cefepime. Eleven *S. aureus* isolates exposed to tetracycline acquired cross-resistance to five additional antibiotics, while three developed resistance to two or three others. Reduced susceptibility to chlorhexidine and resazurin was observed in strains exposed to sublethal concentrations of tetracycline. After exposure to RCE, all tested strains were found to be more resistant to RCE, with MIC values increased by 4- to 32-fold. Most of them showed no significant change in CHX susceptibility, except for three isolates that showed a 4- to 8-fold MIC increase.

Renzoni *et al.* (2017) used CHX as a reference antiseptic in their polyhexanide study. Applying a stepwise exposure by increment method, culturing MRSA strains with increasing concentrations of CHX every two days for 7 to 10 passages, they obtained mutants with 2- to 4-fold increased antiseptic tolerance. In one of the obtained CHX mutants, point mutations led to amino acid changes in the MepA (an efflux pump protein) and PurR (a DNA-binding transcriptional repressor that regulates the expression of several genes involved in the synthesis, metabolism, and transport of purines) proteins. In the second CHX mutant, sequencing revealed mutations in genes (*mprF*, *pldB*, and *glpD*) involved in lipid metabolism resulting in amino acid substitutions. For the obtained MRSA mutants with reduced CHX susceptibility neither cross-resistance with polyhexanide nor with antibiotics was observed.

A total of 177 clinical isolates from early plaque colonizers were exposed to subinhibitory levels of CXG using the gradient method (Auer *et al.* 2022). These isolates included 112 *Streptococcus* spp., 19 *Actinomyces* spp., 20 *Rothia* spp., and 26 *Veillonella* spp. After exposure to the antiseptic, a 2-fold MIC increase was observed for *Veillonella* and *Rothia* isolates, a 2- to 4-fold MIC increase for *Actinomyces* isolates, and a 2- to 8-fold MIC increase for *Streptococcus* isolates. Only mutants showing an 8-fold MIC increase were used for further research. Among them there were mutants resistant to erythromycin and tetracycline, intermediate resistant to penicillin G and ampicillin, and intermediate or resistant to cefuroxime and amoxicillin/clavulanic acid. These isolates were further examined for the presence of antibiotic resistance genes. The antibiotic resistance genes as *MefI* and *tetM* were detected, which correlated with their phenotypic resistance to erythromycin and tetracycline, respectively. In addition, *patA* and *patB* genes were found, which are associated with

resistance to the fluoroquinolone - moxifloxacin; however, the obtained mutants did not show resistance to this antibiotic. The presence of two genes encoding proteins involved in the transposition of the Tn916 transposon was also detected, namely *int-II*, responsible for the production of integrase, and *xis-II*, responsible for the production of excisionase. In addition, these strains showed an increased capacity for biofilm formation (Auer *et al.* 2022).

Früh *et al.* (2022) using the gradient method, examined the effect of repeated exposure to subinhibitory levels of chlorhexidine digluconate on supragingival plaque samples from six healthy volunteers. After 10 sequential passages in CXG, each time selecting the highest concentration still supporting growth, *Streptococcus oralis* and *Granulicatella adiacens* were isolated from the biofilm of these samples. Furthermore, *G. adiacens* exhibited a 4-fold CXG MIC increase and a 2-fold CXG MBC increase, whereas *S. oralis* showed a 2-fold MIC increase and a 4-fold MBC increase. The antibiotic susceptibility of these mutants was then assessed, revealing that *S. oralis* showed decreased susceptibility to erythromycin and increased MIC for clindamycin, amoxicillin, and ampicillin. On the other hand, *G. adiacens* showed reduced susceptibility to erythromycin and clindamycin, as well as increased MICs for penicillin G, tetracycline, cefuroxime, and ciprofloxacin. The study also showed that exposure to CXG for 10 days had no significant effect on the ability of *S. oralis* isolates to form biofilm, while in *G. adiacens* it led to increased biofilm formation.

Spettel *et al.* (2025) performed an *in vitro* study on the effects of long-term exposure of three biocides, CHX, OCT and triclosan, on 96 isolates of *C. albicans* and *Nakaseomyces glabratus* (formerly *Candida glabrata*) using the high-throughput modified increment method. These strains were exposed to increasing concentrations of each biocide for 60 days. No *C. albicans* strain showed changes in sensitivity to CHX, OCT and triclosan after long-term biocide exposure. However, for several *N. glabratus* strains, mutants with reduced sensitivity to CHX (4-fold increase in MIC values) and triclosan (from 4- to 16-fold increase in MIC values) were generated. Furthermore, long-term exposure to CHX, OCT, or triclosan did not induce antiseptic cross-resistance. On the other hand, after prolonged exposure to CHX and triclosan, *N. glabratus* mutants developed resistance to following azoles: fluconazole, posaconazole, voriconazole, itraconazole and isavuconazole, with a 4- to 512-fold increase in MIC values. Whole-genome sequencing of the azole-resistant *N. glabratus* mutants genomes revealed potential gain-of-

function mutations in the transcription factor PDR1, which is responsible for the control of efflux pump genes expression, including *Cdr1p*, *Cdr2p*, and *Snq2p* genes. These mutations identified at positions D261Y, C469R, L936S, G943A, D1082G, and G1088E. Overexpression of the genes encoding these efflux pumps, *Cdr1/2p* and *Snq2p*, has previously been implicated as one of the mechanisms responsible for azole resistance. Furthermore, Spettel *et al.* (2025) demonstrated overexpression of the *CDR1* efflux pump gene in these mutants. In other seven azole-resistant *N. glabratus* mutants that did not have changes in PDR1, mutations in the *PMA1* gene were demonstrated. It is known that PMA1 plays a role of a major regulator of intracellular pH in fungi. The detected mutations may therefore lead to the loss of PDR1 functionality and, further, to a reduction in the intracellular cytosolic pH, which may result in a decrease in the fungal susceptibility to azoles. However, 4 of the 7 PDR1-mutants also showed overexpression of the efflux pump *CDR1* gene.

2.2. Exposure to octenidine and changes in susceptibility profiles

Despite the studies undertaken on the exposure of bacterial and fungal strains to octenidine, only in a few cases mutants with reduced sensitivity to this antiseptic or showing cross-resistance to antibiotics were generated.

Garratt *et al.* (2021) examined how long-term exposure to OCT affects the sensitivity and development of resistance in Gram-negative bacteria present in the waste trap of a hospital sink. During the experiment, water samples were collected from the trap successively at time points T0, T28, T62, T97, and T118 days. After 28 days of exposure to OCT, an increase in tolerance was observed in *P. aeruginosa* (the MIC and MBC values increased from 4 µg/ml to > 64 µg/ml). In *Citrobacter* spp. isolates, a constant 2-fold increase in the MIC and MBC values was observed at subsequent time points starting at T62. *Enterobacter* spp. exhibited cross-resistance to ciprofloxacin, chloramphenicol and ceftazidime, while *Citrobacter* spp. were cross-resistant to ampicillin, piperacillin, ceftazidime, ciprofloxacin, chloramphenicol and meropenem. Additionally, it was examined whether these strains developed tolerance to cetylpyridinium chloride, hexadecylpyridinium chloride monohydrate, benzalkonium chloride, cetyltrimethylammonium bromide, triclosan, chlorhexidine digluconate, and cetrimide. The results showed that *Enterobacter* spp. became more tolerant to all tested biocides except benzalkonium chloride. *Citrobacter* spp. showed increased resistance to most tested biocides,

while *P. aeruginosa* developed the greatest resistance to chlorhexidine. It was also analyzed whether exposure to octenidine influenced the growth rate. A reduction in growth was observed in *Citrobacter* strains isolated at later time points, while the growth rate of *P. aeruginosa* and *Enterobacter* spp. remained unchanged. Virulence testing in the *Galleria mellonella* model revealed a loss of virulence in *Citrobacter* spp., which was not observed in *Enterobacter* spp. and *P. aeruginosa* (Garratt *et al.* 2021). Table III lists and characterizes the microbial mutants obtained after exposure to octenidine.

Shepherd *et al.* (2018) assessed the effectiveness and implications of OCT exposure of *P. aeruginosa* strains in both laboratory and hospital settings. The first study group contained *P. aeruginosa* strains isolated from clinical materials of hospital patients. Adaptation of strains to increasing OCT concentrations was performed in laboratory conditions by the increment method, transferring the obtained cultures to new media with 2-fold higher OCT concentrations every two days for 2 weeks. The second group contained *P. aeruginosa* isolated from a hospital drain trap, exposed to 0.3% OCT bodywash solution four times a day for three months. Water samples for isolation of bacteria were taken from the drain trap at regular intervals. Only one of the first group of clinical strains exposed to OCT exhibited significant changes in antibiotic resistance: a 4-fold increase in gentamicin MIC (up to 32 µg/ml), a 2-fold increase for amikacin (up to 32 µg/ml), a 2-fold increase for tobramycin (up to 8 µg/ml), and a 4-fold increase for colistin (up to 4 µg/ml). This strain also showed an 8-fold increase in OCT MIC and increased tolerance to CHX. In a simulated hospital environment using an automated sink and drain system, an 8-fold increase in OCT MIC (from 4 µg/ml to 32 µg/ml) for the second group of *P. aeruginosa* isolates was recorded. However, after 10 days without biocide bodywash exposure, the OCT MIC values decreased and then returned to 32 µg/ml after 5 days of re-exposure (Shepherd *et al.* 2018).

On the other hand, Spettel *et al.* (2025) did not obtain fungal mutants with altered sensitivity to OCT when they exposed 96 strains of *C. albicans* and *N. glabratus* to this antiseptic for 60 days. Also, none of the strains changed the level of sensitivity to the tested azoles.

2.3. Exposure to alcohol / PVP-I and changes in susceptibility profiles

Shepherd and Parker (2023) investigated how repeated exposure to an antibacterial liquid handwash containing ethyl alcohol (Lifebuoy) can affect bacterial

resistance to antimicrobials and potential cross-resistance to antibiotics. The test was conducted according with EN 1276. Exposure steps were performed repeatedly, reflecting consumer handwash use, over a 4–5 day period. The tested strains included *S. aureus* ATCC 6538, *S. epidermidis* ATCC 14990, *E. coli* ATCC 10536, *E. hirae* ATCC 10541 and *P. aeruginosa* ATCC 15442. It was shown that, even at a 1/100 dilution and a brief handwashing contact time of 10 seconds, the tested strains were unable to survive eight repeat-exposures. In general, repeated exposure to liquid soap did not cause significant changes in antibiotic susceptibility (Table III).

Barakat *et al.* (2022) studied long-term exposure to two commercially available biocidal preparations on the potential development of antiseptic and antibiotic resistance in *S. aureus* ATCC 25923. The first product contained 10% w/v PVP-I and the second named PBM was a mix containing 45% w/w 1-propanol, 30% w/w 2-propanol, and 0.2% w/w mectronium ethyl sulphate. The exposures were performed using increment method. After 10 passages, only a 2-fold increase in PVP-I MIC was noted (from 5,000 µg/ml to 10,000 µg/ml), which decreased to 5,000 µg/ml after another five passages in medium without antiseptic. When the strain was exposed to PBM, a obtained mutant showed a 128-fold increase in PBM MIC (from 664 µg/ml to 85,000 µg/ml) and was still stable after five subsequent passages in medium without biocide. This mutant acquired cross-resistance to cefoxitin, penicillin, ciprofloxacin, and intermediate-level resistance to clindamycin (Table III). Furthermore, the vancomycin MIC value of the PBM-resistant mutant increased 4-fold but the mutant still remained sensitive to this antibiotic.

In additionally, Barakat *et al.* (2022) the effects of short-term exposure to subinhibitory concentrations (1/4 and 1/2 MIC) of two commercially available biocidal preparations on the potential development of virulence in both *S. aureus* ATCC 25923 and PBM-resistant mutant was investigated. Subinhibitory concentrations of PVP-I (1/4 and 1/2 MIC) significantly reduced hemolysin activity (by 7% and 0.28%, respectively) and completely inhibited biofilm formation only in the case of *S. aureus* ATCC 25923. In contrast, subinhibitory concentrations of PBM led to a non-significant decrease in hemolysin activity and a moderate reduction in biofilm activity in both strains. Moreover, the 1/2 PVP-I MIC value significantly downregulated in *S. aureus* ATCC 25923 the expression of *hla* gene responsible for alpha-hemolysin activity, and the following biofilm formulation genes: *ebps*, *eno*, *fib*, *icaA*, and *icaD*.

3. Conclusion

Research has shown that long-term exposing microorganisms to subinhibitory concentrations of antiseptics can reduce their susceptibility to these biocides and, in some cases, lead to the development of cross-resistance to antibiotics. Among the four most commonly used antiseptics (chlorhexidine, octenidine, ethyl alcohol, and povidone-iodine), chlorhexidine has been the most extensively studied and has demonstrated the most significant changes in microbial susceptibility after exposure. Antiseptic products remain generally effective since the concentrations of biocidal substances they contain are at least 100 times higher than the MIC values for most microorganisms. Although their misuse (e.g., inappropriate concentrations, unsuitable surfaces, against inappropriate bioburden, or targeting microorganisms outside the agent's spectrum) poses a serious concern. Improper use can contribute to shifts in microbial drug susceptibility, potentially may leading to clinically relevant consequences. These findings highlight the need to broaden research of antiseptic effectiveness to a wider range of bacterial and fungal species, as well as inclusion of MDR strains with specific drug resistance mechanisms. This applies to both scientific research and research according to the EN standards. Only a detailed understanding of the molecular mechanisms driving altered susceptibility to antiseptics will support the development of effective strategies that minimize the risk of resistance emergence. Responsible use of antiseptics is therefore essential. They should be employed only when clearly beneficial, and overuse must be avoided. Education efforts targeting both the public and healthcare professionals should emphasize the importance of proper disposal of unused or expired antiseptics and their residues.

It is also important to note that biocides are extensively used in agriculture, where they are sprayed in the environment and on vehicles to help limit the spread of infections to animals. In such settings, maintaining sufficiently high biocide concentrations is crucial for preserving their efficacy and minimizing the development of resistance. In line with the One Health concept - which emphasizes the interconnectedness of human, animal, and environmental health - it is vital to implement stricter control over the use of biocides not only in healthcare settings, but also in veterinary and livestock environments. Compliance with current guidelines in all these areas is crucial for effective prevention and controlling the spread of infectious diseases and antimicrobial resistance.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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