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Adhesion of *E. coli* to line HUVEC cel cultures (primary human umbilical vein endothelial cells). Imaging was performed by scanning electron microscopy at 20 kV accelerating voltage, 13.5 mm operating distance, 3.5k magnification

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REVIEW
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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 was 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 destroys microorganisms when applied to a 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 surfaces, 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 monography (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. It's 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 concentration 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 ≥ 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 ≥ 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 ≥ 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 lover 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 ≥ 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, photodermatosis 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/tabl.	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 iodinatum	10%	ointment	for local treatment of burns, wounds, abrasions, trophic ulcers, skin infections
Betadine	povidonum iodinatum	75 mg/ml	solution	for washing hands before surgery and hygienic disinfection of hands
Braunoderm	povidonum iodinatum, 2-propanolum	(1g + 50g)/100g	solution	for disinfection of intact skin before surgery, injections, punctures, catheterization
Jodi Gel	povidonum iodinatum	10%	gel	for disinfecting wounds and skin before surgical procedures, in stomatitis, in primary and secondary local skin infections
PV Jod 10%	povidonum iodinatum	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 remove 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	*S. enterica*	chromosome	triclosan	(Rensch *et al*. 2014)
	LmrS	*S. aureus*	chromosome	benzalkonium chloride	(Kernberger-Fischer *et al*. 2018)
	MdeA	*S. aureus*	chromosome	benzalkonium chloride	(Huang *et al*. 2004)
	MdrL	*L. monocytogenes*	chromosome	benzethonium chloride	(Romanova *et al*. 2006)
	NorA	*S. aureus,* *S. epidermidis*	chromosome	benzalkonium chloride, cetrimide, acriflavine	(Furi *et al*. 2013; Qingzhong *et al*. 2015; Costa *et al*. 2018)
	NorB	*S. aureus*	chromosome	cetrimide	(Qingzhong *et al*. 2015)
	QacA	*S. aureus*	plasmid	chlorhexidine, benzethonium chloride	(Noguchi *et al*. 1999)
	QacB	*S. aureus*	plasmid	chlorhexidine	(Furi *et al*. 2013)
	SmvA	*P. aeruginosa,* *K. pneumoniae*	chromosome	chlorhexidine, octenidine	(Wand *et al*. 2019; Bock *et al*. 2021)
ABC	EfrAB	*E. faecalis,* *E. faecium*	chromosome	chlorhexidine, triclosan	(Lerma *et al*. 2014)
	PatAB	*S. pneumoniae,* *S.pseudopneumoniae*	chromosome	acriflavine	(Robertson *et al.* 2005; Alvarado *et al.* 2017)
RND	AdeABC	*A. baumannii*	chromosome	chlorhexidine, octenidine, benzalkonium chloride	(Meyer *et al*. 2022)
	AcrAB-TolC	*S. enterica,* *E. coli,* *K. pneumoniae*	chromosome	chlorhexidine, triclosan	(Mcmurry *et al.* 1998; Webber *et al.* 2008; Curiao *et al*. 2015)
	AcrEF	*S. enterica*	chromosome	triclosan	(Rensch *et al.* 2014)
	MexAB-OprM	*P. aeruginosa*	chromosome	chlorhexidine[1], triclosan	(Schweizer 1998; Hashemi *et al*. 2019)
	MexCD-OprJ	*P. aeruginosa*	chromosome	chlorhexidine, benzalkonium chloride, triclosan	(Chuanchuen *et al*. 2001; Morita *et al*. 2003)
	MexEF-OprN	*P. aeruginosa*	chromosome	triclosan	(Chuanchuen *et al*. 2001)
	MexXY	*P. aeruginosa*	chromosome	chlorhexidine[2]	(Tag ElDein *et al*. 2021)
	OqxAB	*E. coli*	plasmid	benzalkonium chloride, triclosan	(Hansen *et al.* 2007)
	SdeAB	*S. marcescens*	chromosome	chlorhexidine, benzalkonium chloride	(Maseda *et al.* 2009)
	SmeDEF	*S. maltophilia*	chromosome	triclosan	(Hernández *et al*. 2011)
	TriABC-OpmH	*P. aeruginosa*	chromosome	triclosan	(Fabre *et al*. 2021)

SMR	EmrE	*E. coli*	plasmid	benzalkonium chloride, acriflavine	(Nishino and Yamaguchi 2001)
	KpnEF	*K. pneumoniae*	chromosome	chlorhexidine, triclosan, benzalkonium chloride	(Srinivasan and Rajamohan 2013)
	QacG	*Staphylococcus* spp.	plasmid	benzalkonium chloride,	(Heir *et al.* 1999)
	QacH	*S. saprophyticus*	plasmid	benzalkonium chloride	(Heir *et al.* 1998)
	QacJ	*S. aureus,* *S. simulans,* *S. intermedius*	plasmid	benzalkonium chloride	(Bjorland *et al.* 2003)
	QacZ	*E. faecalis*	plasmid	benzalkonium chloride	(Braga *et al.* 2010)
	Smr	*S. aureus*	plasmid	chlorhexidine, benzalkonium chloride	(Noguchi *et a*l. 1999)
MATE	AbeM	*A. baumannii*	chromosome	triclosan, acriflavine	(Su *et al.* 2005)
	MepA	*S. aureus*	chromosome	chlorhexidine, benzalkonium chloride, cetrimide	(Costa *et al.* 2013)
PACE	AceI	*A. baumannii*	chromosome	chlorhexidine	(Hassan *et al.* 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, [2]increased 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 ≥ 4 μg/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 μg/ml to 1 μg/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 μg/ml, with MIC ≥ 4 μg/ml occurring three times more frequently 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 ≤ 0.25 to ≥ 16 μg/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*, *qacEΔ1* 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 *Enterobacterales* 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 OTC'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-C_3-5), 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 $\log_{10}$ was observed, while 2 μM caused a 5.32 $\log_{10}$ 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-Marin *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 denaturants 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 $\geq 4 \log_{10}$ (Hacioglu *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 $\log_{10}$ 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 $\log_{10}$. 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 $\log_{10}$ 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 (Elekhnawy *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 ≥ 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 × MIC, 1 × MIC, 2 × MIC, and 4 × 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 ≥ 64 μg/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 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 μg/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 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 (Garratt *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 e xposure 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 ($\log_2$ fold change: 3.635), while ST846-OXA48 was characterized by high expression of the *pmrD* ($\log_2$ fold change: 2.36) and *pmrK* ($\log_2$ 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		
Citrobacter 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 *marR* and *envZ*	(Garratt *et al.* 2021)
Enterobacter 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 *malT* and *torA*, D21E mutation in *SmvA*	(Garratt *et al.* 2021)
E. coli	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: *emrAB, ompX, ompA, , gadE, mdtEF, gadABC, cadA, hdeABD, ydeN* downregulation: *mlaA, cdaR, rob, soxS, ompT, ompF*	(Gregorchuk *et al.* 2021)
	ethyl alcohol	no relevant MIC changes	no changes in antibiotic susceptibility	nt[c] / nt	(Shepherd and Parker 2023)
K. pneumoniae	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 *cepA*, mutation Leu82Arg in *PmrB*	(Zhang *et al.* 2019)
		4-fold increase in MIC	32-fold increase in MIC for colistin	nt / overexpressed gene *smvA,* high expression of the *pmrD* and *pmrK*, identification of PemI/PemK TA system, PemK toxin expression reduced biofilm formation	(Bleriot *et al.* 2020)
		4-fold increase in MIC	8-fold increase in MIC for colistin	colonies of irregular shape and rough surfaces / nt	(Hashemi *et al.* 2019)
A. baumannii	chlorhexidine	4-fold increase in MIC	16-fold increase in MIC for colistin	colonies of irregular shape and rough surfaces / nt	(Hashemi *et al.* 2019)

P. aeruginosa	chlorhexidine	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 *et al.* 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 *mexA, mexC, mexE, mexX*, downregulation of *oprD*	(Zheng *et al.* 2022)
		2- to 22-fold increase in MIC	no changes in antibiotic susceptibility	nt / nt	(Karpiński *et al.* 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 *mexX*	(Tag ElDein *et al.* 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 *smvR* (TetR regulator)	(Garratt *et al.* 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 *G. mellonella* model, three showed a decreased growth rate / nt	(Shepherd *et al.* 2018)
		3- to 12-fold increase in MIC	no changes in antibiotic susceptibility	nt / nt	(Karpiński *et al.* 2025)
	povidone-iodine	4-fold increase in MIC	no changes in antibiotic susceptibility	nt / nt	(Karpiński *et al.* 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)
E. hirae	ethyl alcohol	no relevant MIC changes	4-fold increase in MIC for gentamicin	nt / nt	(Shepherd and Parker 2023)
S. aureus	chlorhexidine	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 *et al.* 2016)
		2- to 4-fold increase in MIC	no changes in antibiotic susceptibility	nt / mutations in *mepA, purr, pldB, glpD,* and *mprF*	(Renzoni and François *et al.* 2017)
	povidone-iodine	2-fold increase in MIC	nt	inhibition biofilm formation, reduced hemolytic activity / downregulation of *icaA, icaD, eno, epbs, fib, hla*	(Barakat *et al.* 2022)

S. epidermidis	ethyl alcohol	no relevant MIC changes	no changes in antibiotic susceptibility	nt / nt	(Shepherd and Parker 2023)
S. oralis	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 *et al.* 2022)
Streptococcus 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*: tetM, patA, patB, mefI, pbpX2, int, xis*	(Auer *et al.* 2022)
G. adiacens	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 *et al.* 2022)
C. albicans	octenidine	no relevant MIC changes	no changes in antibiotic susceptibility	nt / nt	(Spettel *et al.* 2025)
	chlorhexidine	no relevant MIC changes	no changes in antibiotic susceptibility	nt / nt	(Spettel *et al.* 2025)
N. glabratus	octenidine	2-fold increase in MIC	no changes in antibiotic susceptibility	nt / nt	(Spettel *et al.* 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 *PDR1*, mutations in *PMA1*, overexpression of *CDR1*	(Spettel *et al.* 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-dglutamate 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. galbratus* 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 mecetronium 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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AKKERMANSIA MUCINIPHILA IS ASSOCIATED WITH HUMAN HEALTH: WHAT SHOULD WE KNOW?

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Akkermansia muciniphila is a gut bacterium that has recently attracted considerable attention in microbiota research. Its presence in the gut is associated with improved metabolic health, enhanced gut barrier integrity, and modulation of the immune system. However, potential risks related to its abundance under certain pathological conditions have also been noted. As *A. muciniphila* emerges as a candidate for next-generation probiotics, evaluating whether current data support its therapeutic use is crucial. In this review, we analyze the available literature to outline the beneficial effects of *A. muciniphila* on the host and critically assess its potential as a probiotic.

1. Introduction. 2. *Akkermansia muciniphila* in the human population. 3. *Akkermansia muciniphila* is a mucin specialist. 4. The protective role of *Akkermansia muciniphila* in diseases. 5. The role of *Akkermansia muciniphila* in obesity prevention. 6. *Akkermansia muciniphila* - potential as a probiotic. 7. Conclusion.

Keywords: *Akkermansia muciniphila*, disease, microbiota, probiotics.

1. Introduction

Akkermansia muciniphila was first isolated from the faeces of a healthy adult Caucasian female in 2004 as the first known member of the *Akkermansia* genus and the only isolated member of the Verrucomicrobiota phylum (Derrien et al. 2004). The anaerobic, Gram-negative, non-motile, and non-spore-forming bacterium colonizes the intestines and nasopharynx of humans and other animals (Derrien et al. 2004). Other environments it inhabits include the appendix, the pancreas (in pathological conditions), human breast milk, and human blood samples (Geerlings et al. 2017). Since then, other members of the genus have been found inhabiting the human gut (Kobayashi et al. 2018). *Akkermansia* is a genus that has recently attracted much attention due to its probiotic effects and possible role in bowel disease treatment (approved by the European Food Safety Authority (EFSA).

2. *Akkermansia muciniphila* in the human population

A. muciniphila is an early colonizer of the human gut that reaches an abundance similar to or slightly lower than that in adults within the first year of life and then decreases in the elderly. The abundance appears to be higher in formula-fed than in breast-fed infants, and increases once breast-feeding stops (Azad et al. 2018). A Chinese study found a colonization rate of 51-74% in southern China and identified 22 strains within the studied population (Guo et al. 2016). Abundance and colonization rate can vary between countries, and the composition of the microbiota is influenced by diet and genetic factors (Grześkowiak et al. 2012). Nonetheless, the consensus is that *A. muciniphila* is a common and stable part of the human gut microbiota. *A. muciniphila* growth can be stimulated by diet, with one study demonstrating that dietary polyphenols from grapes can dramatically promote

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its growth in mouse models (Roopchand et al. 2015). Another study demonstrated that fucoids from brown seaweed increased the abundance of *Akkermansia* in mice with metabolic syndrome induced by a high-fat diet (Qingsen et al. 2017).

3. *Akkermansia muciniphila* is a mucin specialist

One of the key characteristics of *A.muciniphila* is the ability to degrade mucins and use them as an energy source. Mucins are high-molecular-weight glycoproteins continuously secreted by goblet cells, which constitute a significant component of the intestinal mucus and form the protective mucus layer. Of the 21 different mucins identified, mucin 2 (MUC2) is the predominant component of the colonic mucus layer and acts as its structural skeleton (Song et al. 2023). The mucus layer serves as the first line of defense, protecting the epithelium from inflammation and infection. Disruption of the mucus layer is an important factor in the development of intestinal diseases, including inflammatory bowel disease (IBD) and colorectal cancer. The mucus barrier maintains homeostasis by stimulating the growth of appropriate microbiota and preventing pathogens from contacting the epithelium (Song et al. 2023).

Mucin-degrading bacteria produce glycosyl hydrolases (GHs), specialized enzymes that enable them to break down mucins. The *A. muciniphila* genome contains genes encoding nine different GH families (Glover et al. 2022). It can utilize different combinations to hydrolyze up to 85% of mucin structures, allowing it to use mucins as its sole carbon source (Glover et al. 2022). As a result, *A. muciniphila* is often considered one of the most important mucin degraders in the human microbiota. The metabolism of mucins by *A. muciniphila*, in addition to the action of other bacterial species, releases monosaccharides, oligosaccharides, and short-chain fatty acids into the intestinal environment, thereby contributing to the modulation of intestinal homeostasis (Belzer et al. 2012). Although *A. muciniphila* degrades mucin, it does so in a controlled and selective manner, which (1) stimulates the production of new, healthy mucus, (2) supports the regeneration of the mucus and epithelial barrier, and (3) reduces inflammation and strengthens mucosal immunity (Si et al. 2022). As such, its presence in the gut microbiota is associated with better metabolic, immune, and barrier health (Table 1).

Table 1. *Akkermansia muciniphila* is positively associated with improved metabolic profiles, enhanced mucosal immunity, and a robust epithelial barrier.

Mechanism	**Effect**	**Reference**
Mucin degradation + mucus stimulation	Promotes goblet cell proliferation and maintains mucus thickness	Si et al. (2022)
Barrier reinforcement	Enhances tight junctions (ZO1, occludin, claudins), and TER ↑	
Immune modulation	↓ proinflammatory cytokines, ↑ IL-10, and ↑ Tregs	

IL-10 – interleukin-10; TER – transepithelial electrical resistance; Tregs – regulatory T cells; ZO-1 – zonula occludin-1.

4. The protective role of *Akkermansia muciniphila* in diseases

The primary reported benefits of *A. muciniphila* are associated with alleviating symptoms or preventing gastrointestinal disease, with a primary focus on Inflammatory Bowel Disease IBD. IBD refers to a group of diseases that cause inflammation of the bowel, with the primary types being ulcerative colitis (UC) and Crohn's disease (CD). Symptoms may include diarrhea, abdominal pain, fatigue, nausea, and weight loss. Significant evidence suggests a correlation between *A. muciniphila* and the development of IBD, although its nature remains under discussion. In a mouse study,

treatment with *A. muciniphila* for five weeks reduced inflammation caused by chemically induced colitis (Yilmaz et al. 2024). Another mouse model found that *A. muciniphila* improved clinical parameters, including spleen weight, colon inflammation index, colon histological score, and regulation of pro-inflammatory cytokines, with varying activity levels among strains (Zhai et al. 2019). In addition, *A. muciniphila* supplementation reduced serum and tissue inflammatory cytokines and chemokines in mice, along with reduced weight loss, improved histological scores, and enhanced barrier function (Bian et al. 2019).

The role of *A. muciniphila* in the prevention of IBD can be inferred from its reduced presence in IBD patients, with UC and CD exhibiting lower colonization rates and abundance compared to healthy individuals, both of which increase significantly after washed microbiota transplantation (Qu et al. 2021). Additionally, *A. muciniphila* was lower in patients with active UC compared to those with quiescent UC and healthy individuals (Zhang et al. 2020). The same study identified a reduction of sulfated mucins in the mucus of IBD patients as a potential cause of *A. muciniphila* reduction (Zhang et al. 2020).

Investigations into the mechanisms underlying the anti-inflammatory effects of *A. muciniphila* are ongoing. Aside from the well-known anti-inflammatory effects of short-chain fatty acids (SCFA) produced by the human microbiota, there have been reports that one of the main surface proteins of *A. muciniphila* (Amuc_1100) could play a crucial role (Wu et al. 2019).

The anti-inflammatory effect of *A. muciniphila* might also be beneficial for patients with Parkinson's disease (PD). Indeed, an *A. muciniphila* treatment alleviated artificially induced PD in mice, including neuroinflammation and motor dysfunction, while promoting neurogenesis (Qiao et al. 2024). However, the evidence for the beneficial effects *of A. muciniphila* remains inconclusive, with some reports not aligning with the previously mentioned results. One such study detected an increase in *A. muciniphila* in patients with colorectal cancer (Weir et al. 2013), suggesting that the relationship between *A. muciniphila* and host health might be more complex.

5. The role of *Akkermansia muciniphila* in obesity prevention

Another area in which *A. muciniphila* is heavily investigated for its beneficial effects is obesity, with the bacterium's anti-obesity effects demonstrated in several studies. An analysis of data from the American Gut Project has found an association between a higher abundance of *A. muciniphila* and a lower risk of obesity (Zhou et al. 2020). A randomized controlled trial reported that *A. muciniphila* supplementation reduced obesity, though the effects appear to be limited to individuals with a low baseline abundance of the bacterium (Zhang et al. 2025). In this regard, the reduction of *A. muciniphila* was associated with the development of atherosclerosis induced by a high-fat Western diet in apolipoprotein E knock-out mice (Li et al. 2016). Meanwhile, daily administration of *A. muciniphila* has been shown to prevent weight gain, hyperphagia, and dysglycemia caused by the dietary emulsifiers carboxymethylcellulose and polysorbate (Daniel et al. 2023).

Investigations into the anti-obesity mechanisms of *A. muciniphila* demonstrated that the species can alleviate the negative effects of interferon gamma (IFNγ) on glucose tolerance (Greer et al. 2016). Another potential mechanism of action involves Amuc_1100, which has some of the same effects as the live bacterium when purified or applied as part of pasteurized *A. muciniphila* (Anhê et al. 2017). It is very likely that the effects of *A. muciniphila* on obesity are not centred on a single mechanism, but result from several separate effects in conjunction with other members of the human gut microbiota. Furthermore, many studies have involved mice fed a high-fat diet, so the impact on different sources of obesity should still be investigated.

6. *Akkermansia muciniphila* - potential as a probiotic

As described previously, *A. muciniphila* has several potential benefits for human health. However, it is worth discussing whether it can be used as a probiotic. Aside from providing health benefits to the host, a good probiotic should be considered safe for human consumption, and it should be able to survive long enough in storage and after consumption to reach the gut. A toxicological analysis of pasteurized *A. muciniphila* did not reveal any mutagenic, clastogenic, or aneugenic effects, nor did it reveal any adverse neurobehavioural or pathological effects that would undermine its use as a food additive (Druart et al. 2021). A comparative analysis of *A. muciniphila* and the commonly used probiotic bacterium *Lactobacillus rhamnosus* GG revealed comparable levels of auto-aggregation, co-aggregation,

hydrophobicity, and antimicrobial activity, but a higher level of antibiotic resistance in *A. muciniphila*. It is generally recommended that probiotic bacteria have a low level of antibiotic resistance to prevent potential horizontal gene transfer. However, the presence of resistance genes associated with transferable genetic elements has not been reported in *A. muciniphila* (Cozzolino et al. 2020).

Methods for cultivating *A. muciniphila* have improved since its initial discovery. Using mucin in growth medium is costly and inconvenient, so the use of alternatives has been investigated. One study identified glucose or N-acetylglucosamine (a component of mucins) as a good source of carbon, and tryptone as a reliable source of nitrogen (Wu et al. 2024). Another study identified galactose, sialic acid, lactose, and chitosan as factors significantly promoting *A. muciniphila* growth (Meng et al. 2024).

7. Conclusions

A. muciniphila supplementation provides significant benefits for patients suffering from IBD. Its relationship with obesity seems to be more complex, though studies agree that it alleviates symptoms associated with a high-fat diet, which is common in Western countries. As a natural member of the human microbiota, *A. muciniphila* is generally considered safe, which is supported by evidence, and has been approved by EFSA. There are also no technical obstacles to its use as a commercial probiotic. *A. muciniphila*'s general functions are summarised in Figure 1.

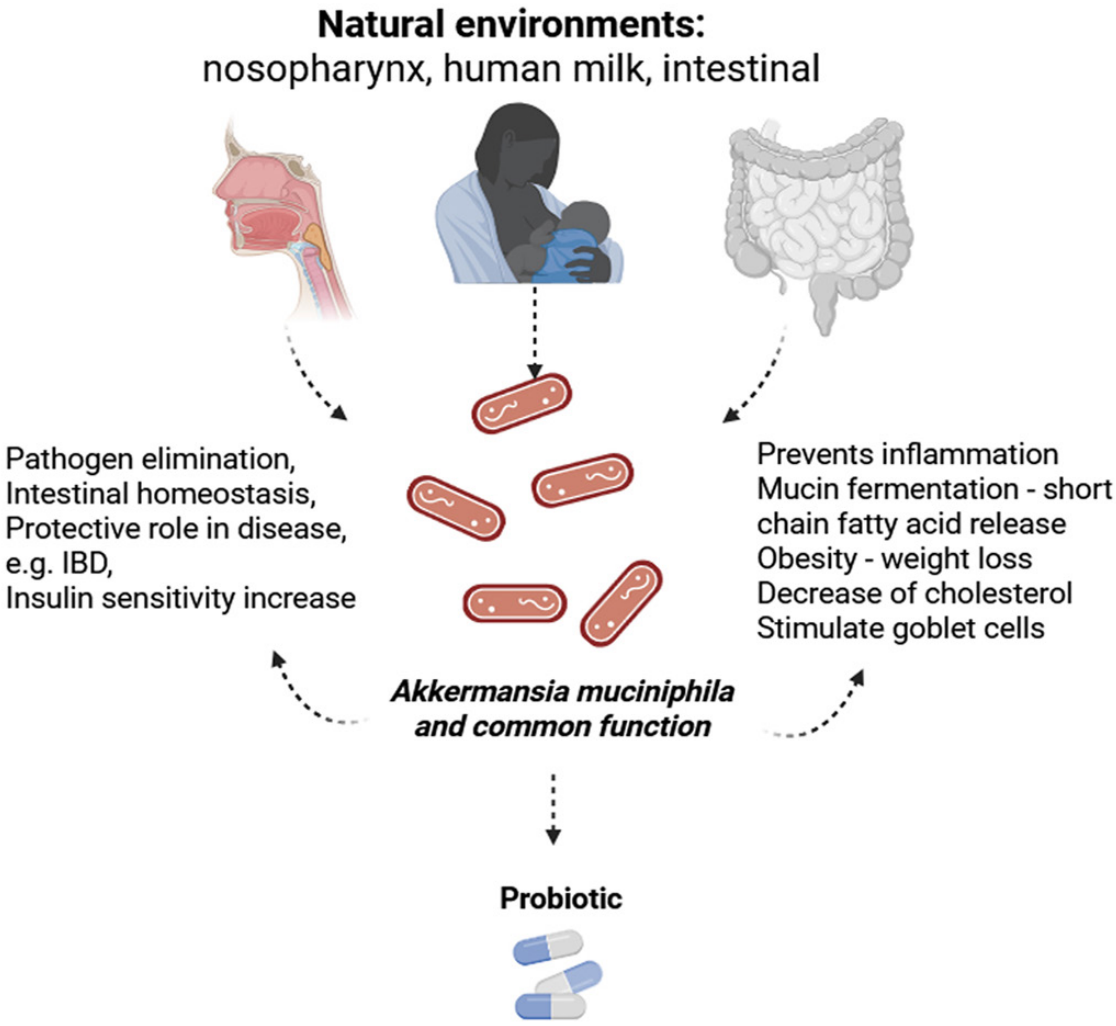

Figure1. Summary of the role of *A.muciniphila* in health and disease. Illustration created using Biorender (www.biorender.com). **Agreement number:** CA28PV7H2E

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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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REVIEW
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LINKING GUT MICROBIOTA AND IRRITABLE BOWEL SYNDROME (IBS): A REVIEW

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Abstract: Irritable bowel syndrome (IBS) is a chronic gastrointestinal disorder with an increasing global prevalence. The International Classification of Diseases (ICD) system typically categorizes IBS into four subtypes based on symptomatology. The objective of this review is to provide a concise synthesis of the most current information regarding IBS, encompassing widely accepted diagnostic criteria, etiology, epidemiological data and the significance of gut microbiota (GM) in pathogenesis of this disorder. Additionally, it will explore future perspectives. Recent studies have demonstrated that the GM in healthy individuals primarily consists of four main bacterial phyla: *Firmicutes* spp., *Bacteroidetes* spp., *Actinobacteria* spp., and *Proteobacteria* spp. Dysbiosis or an imbalance in these bacteria may be a contributing factor to the IBS development. It is imperative to acknowledge the multifaceted role of the GM in several essential biological processes, including: immunomodulation, intestinal barrier integrity, gut microbiota-gut-brain axis (GBA) or nutrient absorption. The composition of GM is subject to variation depending on the IBS subtype. Many therapeutic strategies have been devised for the treatment of patients with IBS, comprising antibiotics, probiotics, prebiotics, synbiotics and fecal microbiota transplantation (FMT). Although FMT has shown promise, clinical trials outcomes remain still inconsistent. Dietary interventions and psychological support are also vital components of IBS management.
Despite the advances in understanding the GM-IBS relationship, there is still a lack of knowledge regarding specific microbial markers for each IBS subtype. Consequently, a definitive microbiota pattern has yet to be delineated. However, emerging evidence underscores the microbiome's role in IBS pathophysiology.

1. Introduction. 2. Gut microbiota. 3. Epidemiology of IBS. 4.The role of gut microbiota in the pathogenesis of irritable bowel syndrome. 4.1. Gut microbiota metabolic products. 4.2. Mucosal immune regulation. 4.3. Intestinal barrier dysfunction. 4.4. Gut microbiota-gut-brain axis. 5. Changes in the gut microbiota composition in dependence on subtype of irritable bowel syndrome. 6. Therapeutic approach in irritable bowel syndrome. 6.1. Diet. 6.2. Antibiotics. 6.3. Probiotics, prebiotics, synbiotics, postbiotics. 6.4. Fecal microbiota transplantation. 6.5. Mind-body therapies. 7. Limitations of current therapies. 8. Future perspectives. 9. Conclusions.

Keywords: gut microbiota/microbiome, IBS, irritable bowel syndrome

1.Introduction

Irritable bowel syndrome is one of the most common functional, gastrointestinal disorder (FGID) characterized by occurrence of following symptoms: bloating, discomfort, abdominal pain, abnormal stool characteristics, changes in bowel habits (constipation or diarrhea). Also symptoms not associated with digestive system were reported including: chronic pelvic pain, temporomandibular joint disorder, fibromyalgia and chronic fatigue syndrome (Cheng et al. 2024; Aggeletopoulou and Triantos 2024; Li et al. 2024). As it was mentioned above, IBS is classified as FGID what means that symptoms (particularly gastrointestinal) cannot be described in the context of structural or metabolic abnormalities (Shaikh et al. 2023). This disorder might

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be divided into four subtypes: constipation-predominant (IBS-C), diarrhea-predominant (IBS-D), mixed (IBS-M), and unsubtyped (IBS-U) according to the Rome IV 2016 (Palsson et al. 2016). Also Bristol Stool Form Scale (BSFS) plays a role in the determination of IBS subtype. This scale assumes characterization of stool consistency from hard to soft based on the scale 1-7 (Shaikh et al. 2023). IBS affects approximately 10-20% population and negatively impacts on the patient's life quality including psychological issues (Pittayanon et al. 2019). It was reported that among patients with this disorder occurred such mental health problems as for example anxiety, depression, suicidal thoughts or work productivity impairment. Moreover, patient with IBS annualy often spend much money on medical care (Aggeletopoulou and Triantos 2024; Chong et al. 2019). Currently, there are no diagnostics criteria and IBS is diagnosed based on mainly patient's symptoms, medical history and by using imaging methods routinely used in gastroenterological practice like endoscopy (Cheng et al. 2024). There are also no biomarkers or specific laboratory tests which could be helpful in the diagnosis and management of this gastrointestinal disease (Shrestha et al. 2022). It highlights that IBS is a challenge in the clinical practice. The pathophysiology of IBS is intricate and not yet fully elucidated. It is widely accepted that this phenomenon arises from disruptions in the complex interactions between the gastrointestinal system and the central nervous system. These disturbances have been hypothesised to result in visceral hypersensitivity, intestinal motility disorders and abnormal signal processing in the central nervous system. The predominant pathophysiological mechanisms of IBS are (a) microbiological or functional disorders of the brain-gut axis, resulting from bacterial overgrowth or disrupted communication, which affects the functioning of the gastrointestinal tract; and (b) altered gastrointestinal motility disorders (abnormal contractions leading to diarrhoea, constipation or alternating periods of both); (c) visceral hypersensitivity associated with pain and discomfort, even in the absence of peristaltic movement; (d) disorders in the intestinal immune system (intestinal inflammation), including excessive immune stimulation or hypersensitivity to food allergens; (e) intestinal dysbiosis, i.e. an imbalance of intestinal microorganisms, both qualitative and quantitative; (f) psychological factors, including prolonged or acute stress, other psychological disorders (low mood, anxiety symptoms, depression, grief), as well as adverse childhood experiences (ACE), which may exacerbate the symptoms of irritable bowel syndrome (IBS); (g) genetic predisposition; (h) previous intestinal infections resulting in permanent alterations in the functioning of the gastrointestinal tract (post-infectious reactivity); (i) a diet that is particularly rich in Fermentable Oligosaccharides, Disaccharides, Monosaccharides, And Polyols (FODMAPs) can trigger symptoms in individuals diagnosed with IBS (Cheng et al. 2024; Li et al. 2024; Almonajjed et al. 2025).

A significant number of authors have considered also the psychosomatic basis of IBS. The manifest symptoms of the digestive system are not necessarily directly related to the pathology of this system itself or the physiological changes that occur in the intestines. It is frequently cited that adverse childhood experiences (ACEs) resulting from severe traumatic events during childhood or adolescence, experiences of extreme poverty, illness in close family members, or war are often mentioned in this context. It is acknowledged that early childhood trauma has the capacity to influence the development of IBS through two primary neurobiological mechanisms. Firstly, there is the dysregulation of the hypothalamic-pituitary-adrenal axis, which can lead to dysregulation of intestinal motility and intestinal dysbiosis. Secondly, there is the disturbance of the brain-gut axis, which can result in, for example, misprocessing of information from the intestines, hypersensitivity and a low pain threshold. The combination of all functional gastrointestinal symptoms of IBS with stress (via neural, hormonal and immune signalling) has been shown to cause further exacerbation of gastrointestinal symptoms, resulting in positive feedback (Almonajjed et al. 2025; Chong et al. 2019; Staudacher et al. 2023).

The purpose of this concise review is to explore the underlying causes of discomfort and the associated symptoms that contribute to the development and perpetuation of this multifaceted disorder. The primary objective of the present review is to emphasise the significance of the GM in the etiopathogenesis and progression of IBS.

2. Gut microbiota

The intestinal microbiota/microbiome comprises bacteria, viruses, protozoa and fungi, all of which play a vital role in maintaining the health of the host. These microorganisms play a pivotal role in a multitude of functions that are indispensable for the proper

functioning of the human organism. Such functions include drug and nutrient metabolism, protection against pathogens and modulation of the immune response (Aggeletopoulou and Triantos 2024; Shaikh et al. 2023). The composition of the intestinal microbiota is influenced by several environmental factors, including age, sex, ethnicity and diet or geographical localisation (Shaikh et al. 2023). The preponderance of bacteria in the intestinal ecosystem has led to the nomenclature of this complex as GM (Cheng et al. 2024).The development of GM has been observed since early childhood (Almonajjed et al. 2025). In the context of healthy individuals, the predominant phyla include *Bacteroides* spp., *Clostridium* spp., *Bifidobacterium* spp. and *Lactobacillus* spp. (Cheng et al. 2024; Almonajjed et al. 2025; Shaikh et al. 2023; Menees and Chey 2018). It is widely accepted that bacteria present in the intestines can be categorised into two distinct groups: namely, beneficial bacteria and pathogenic bacteria. The former are primarily represented by the phyla mentioned above: *Bacteroides* spp., *Clostridium* spp., *Bifidobacterium* spp. and *Lactobacillus* spp. It is now evident that these bacteria are involved in facilitating a multitude of beneficial processes within the human organism, including the synthesis of vitamins (e.g. K_2, B_1, B_2, B_6, B_7, B_9, and B_{12}) and the production of (SCFAs, e.g. acetate, butyrate, and propioniate), amino acids, carbohydrates, and lipids, the absorption of important ions (e.g. magnesium, iron, and zinc), the biosynthesis of cholesterol from bile acids (BAs), and the protection against different pathogens by the production of antimicrobial substances (e.g. bacteriocins and lactic acid) (**Table 1**). The second group of GM comprises opportunistic bacteria with pathogenic potential, including enteric bacteria such as *Salmonella* spp. and *Escherichia coli*. These bacteria are responsible for the production of harmful substances that can lead to various pathological conditions. Furthermore, opportunistic bacteria, including *Enterococcus* spp. and Enterobacterales, have been identified as significant contributors to diseases, particularly in individuals with compromised immune systems (Cheng et al. 2024). The composition of the gut microbiome can be studied using a variety of molecular methods, including terminal restriction fragment length polymorphism, 16S ribosomal RNA (rRNA) gene sequencing, quantitative polymerase chain reaction (qPCR), fluorescent *in-situ* hybridization, bacterial culture or microarrays (Pittayanon et al. 2019).

Table 1: Examples of gut microbiota phyla and taxa, along with an analysis of their role in the functioning of the human organism based on (Almonajjed et al. 2025; Mamieva et al. 2022)

Phylum	Taxa	Role
Firmicutes	*Enterococcus, Ruminococcus*, *Clostridium*, *Lactobacillus*, *Faecalibacterium*, *Roseburia*, *Eubacterium*	metabolism of amino acids, carbohydrates and lipids, the transformation of BAs and the biosynthesis of cholesterol, the synthesis of vitamins (K_2, B_1, B_2, B_6, B_7, B_9 and B_{12}) support the integrity of the intestinal epithelial barrier and protection against enteric infections
Bacteroidetes	*Bacteroides*, *Prevotella*	immunomodulation, appetite regulation
Actinobacteria	*Bifidobacterium*, *Corynebacterium*	vitamin synthesis, BAs metabolism, protection against infections
Proteobacteria	*Shigella*, *Escherichia, Desulfovibrio*	amino-acids metabolism

3. Epidemiology of IBS

The global prevalence of IBS varies depending on the diagnostic criteria used and the geographical location. Based on Rome IV criteria, the global prevalence of IBS is estimated at 3.8%. The highest prevalence is found in South America (21%), while the lowest in Southeast Asia (7%) (Oka et al. 2020). IBS is more prevalent among women than men, with an approximate female-to-male ratio of about 2:1 in Western countries (Sperber et al. 2021; Lovell and Ford 2012). Women more frequently report IBS-C, while men more often

report IBS-D (Lovell and Ford 2012). Onset typically occurs before age 50, often in late adolescence or early adulthood (Canavan et al. 2014). IBS is more frequently reported in Western countries, although underdiagnosis in low- and middle-income countries due to lack of access to healthcare and cultural differences in symptom reporting may mean that the true prevalence is underestimated (Oka et al. 2020; Sperber et al. 2021). A higher prevalence is often observed in urban areas and among individuals with a higher level of education and a higher socioeconomic status, potentially due to increased access to healthcare and health-seeking behaviour (Hungin et al. 2005).

4. The role of gut microbiota in the pathogenesis of irritable bowel syndrome

The role of the GM in the development of IBS is a subject that has attracted considerable interest from the scientific community. This group of microorganisms plays a number of pivotal roles in a variety of processes, including the production of different metabolites from absorbed nutrients in the intestines, the regulation of GBA, mucosal immune regulation, intestinal barrier dysfunction, gastrointestinal motility and visceral sensitivity (Almonajjed et al. 2025; Mamieva et al. 2022; Cheng et al. 2024).

4.1. Gut microbiota metabolic products

The development of IBS is significantly impacted by GM, which produces various metabolic factors, including SCFAs, neurotransmitters (e.g. serotonin), lipopolysaccharides, peptidoglycans, BAs and signalling molecules. These products are derived from nutrients absorbed in the intestines and subsequently metabolised by GM through a series of metabolic processes. It is evident that all these factors collectively influence the manifestation of IBS symptoms (Cheng et al. 2024).

Bile acids are synthesised in the human intestine by a variety of bacterial phyla, including: *Bacteroides*, *Clostridium*, *Lactobacillus*, *Listeria*, and *Bifidobacterium*. It has been demonstrated that alterations in the concentration of BAs have been demonstrated to induce cytotoxic effects, encompassing apoptosis, necrosis and DNA damage. These alterations are considered a primary contributing factor to the development of IBS. An imbalance in the synthesis of BAs has been particularly observed among patients with IBS-D, who also exhibit decreased levels of bacteria belonging to the Ruminococcaceae family (Aggeletopoulou and Triantos 2024; Shrestha et al. 2022).

SCFAs, including butyrate and propionic acids, are a by-product of the anaerobic metabolism of carbohydrates and play a pivotal role in maintaining intestinal barrier integrity and regulating immune functions (Cheng et al. 2024; Aggeletopoulou and Triantos 2024). In addition to their role in metabolism, SCFAs have also demonstrated the capacity to exhibit anti-inflammatory activity. Reduced levels of SCFAs have been observed primarily among patients diagnosed with IBS-C (Cheng et al. 2024). SCFAs play a crucial role in the synthesis of serotonin, a neurotransmitter of significant importance within the central nervous system (CNS). Serotonin is synthesised from tryptophan by enterochromaffin (EC) cells or directly by bacteria. This neurotransmitter is responsible for gut peristalsis, regulation of secretion and vasodilator function (Aggeletopoulou and Triantos 2024; Shaikh et al. 2023; Mamieva et al. 2022). Increased serotonin synthesis has been linked to diarrhea (IBS-D), while reduced serotonin levels have been associated with IBS-C (Shaikh et al. 2023; Mamieva et al. 2022). Additionally, SCFAs have been identified as modulators of glucagon-like peptide 1 (GLP-1) secretion by intestinal L-cells. The bacteria representing *Clostridium* spp., *Bacteroides* spp. and *Ruminococcus* spp. are the main contributors to this process (Mamieva et al. 2022). The primary function of GLP-1 is to reduce motility in the antrum, duodenum and jejunum (Mamieva et al. 2022). Levels of this factor are reduced in patients diagnosed with IBS-C (Li et al. 2017). Furthermore, SCFAs have been identified as promising biomarkers for IBS (Cheng et al. 2024).

It is evident that components of the bacterial cell wall, such as lipopolysaccharides (LPS) and peptidoglycans (PGs), play a pivotal role in the activation of the immune system through the recognition process by Toll-like receptors (TLRs). Thereafter, immune cells secrete various cytokines and mediators, which are instrumental in the process of immune response. Of particular significance is the secretion of histamine by mast cells, a process that is implicated in the occurrence of gut permeability, mucosal inflammation and visceral hypersensitivity, which are characteristic of IBS symptoms (Cheng et al. 2024; Aggeletopoulou and Triantos 2024).

In conclusion, it is evident that GM metabolic products play a pivotal role in the regulation of gastrointestinal functions, immunomodulation, and the synthesis

of factors necessary for the normal functioning of the human organism. Conversely, there is also evidence to suggest that metabolic products have also been associated with the symptoms and progression of IBS.

4.2. Mucosal immune regulation

The immune response in patients diagnosed with IBS has been shown to be dysregulated. This has been linked to the migration of immune cells, primarily mast cells, to the intestinal mucosa, leading to the onset of inflammation (Aggeletopoulou and Triantos 2024; Mamieva et al. 2022). In response to the recognition of bacterial antigens by TLRs, mast cells secrete a range of immune response mediators, including histamine, tryptamine, prostaglandins, serotonin and proteases. The mediators in question have been identified as playing a crucial role in immunotolerance (Cheng et al. 2024; Aggeletopoulou and Triantos 2024). The aforementioned mediators have been linked to the occurrence of IBS symptoms, visceral hypersensitivity, altered pain threshold and intestinal barrier dysfunction (Aggeletopoulou and Triantos 2024; Almonajjed et al. 2025; Mamieva et al. 2022). Furthermore, an additional finding of significance is the observation that tryptase release is a causative factor in the reduction of expression of tight junction proteins, thereby increasing gut permeability (Almonajjed et al. 2025; Mamieva et al. 2022). A plethora of studies have identified elevated levels of various immune mediators, including IL-6, IL-8, IL-12, IL-1β and tumour necrosis factor-α (TNF-α), in patients with IBS. Conversely, a paucity of research has been observed with regard to IL-10 levels, which have been shown to be reduced in such cases (Aggeletopoulou and Triantos 2024; Mamieva et al. 2022). The immune response is influenced by the production of metabolites by several phyla. Bacteria belonging to the phylum *Firmicutes* are responsible for the production of butyrate, which is involved in the differentiation of regulatory T-cells (Treg.) (Mamieva et al. 2022). *Lactobacillus* spp. transform tryptophan into indole-3-aldehyde, which leads to the activation of the aryl hydrocarbon receptor (AHR). The AHR is involved in the regulation of the number of intraepithelial lymphocytes and IL-22 production (Almonajjed et al. 2025; Mamieva et al. 2022). Furthermore, *Lactobacillus rhamnosus*, *Lactobacillus casei* and *Bifidobacterium breve* have been observed to induce IL-4 and IL-10 production, while *L. reuteri* and *L. plantarum* have been shown to downregulate the expression of TNF-α (Mamieva et al. 2022). Butyrate-producing *Faecalibacterium prausnitzii* is a bacterial species that has been shown to be responsible for anti-inflammatory activity through inhibition of IL-8 synthesis, activation of regulatory T-cells (Treg) and increased secretion of IL-10 (Almonajjed et al. 2025). In patients diagnosed with post-infectious IBS (PI-IBS), an increased abundance of *Bacteroidetes* and a concurrent decrease in *Clostridiales* have been observed. These changes have been shown to correlate with elevated levels of cytokines (IL-1β and IL-6), which are involved in inflammatory processes (Aggeletopoulou and Triantos 2024).

The scientific literature indicates that GM play a crucial role in regulating immune responses, and that they are involved in the pathophysiology of IBS, with a consequent effect on the severity and symptoms of the condition.

4.3. Intestinal barrier dysfunction

The intestinal barrier plays a pivotal role in preserving gut homeostasis, a process that involves the prevention of antigen migration to the mucosa and the subsequent development of mucosal inflammation (Mamieva et al. 2022).The intestinal barrier dysfunction is a multifaceted condition, with involvement of both metabolic and immune pathways (Mamieva et al. 2022). A salient feature of intestinal barrier dysfunction is its high prevalence among patients diagnosed with IBS-D (Almonajjed et al. 2025). The underlying causes of this increased gut permeability are multifaceted, including, but not limited to, reduced expression of tight junction proteins, such as occludin, claudins, and zonula occludens-1, in the duodenum, colon, and jejunum (Cheng et al. 2024; Mamieva et al. 2022; D'Antongiovanni et al. 2020). The role of GM in maintaining intestinal integrity is significant, with bacteria from the phylum *Firmicutes* (*Eubacterium* spp., *Clostridium* spp., *Ruminococcus* spp. and *Faecalibacterium* spp.) producing SCFAs. Recent studies have demonstrated the pivotal function of these SCFAs in modulationg the expression of claudins (3 and 4) and occludins (Almonajjed et al. 2025; Mamieva et al. 2022). The production of E-cadherin and zonula occludens-1 is stimulated by genera such as *Clostridium* spp., *Enterococcus* spp., *Streptococcus* spp. and *Lactobacillus* spp., which are involved in the production of polyamines (Almonajjed et al. 2025; Mamieva et al. 2022). Tight junction protein ZO-2 plays a crucial role in maintaining the intestinal barrier function, with its expression being

stimulated by bacteria *E. coli* (Cheng et al. 2024). Probiotic bacteria, typified by *Lactobacillus* spp. and *Bifidobacterium* spp., have been demonstrated to excert a beneficial influence on the intestinal barrier function of patients with IBS through the inhibition of increased permeability and the regulation of secretion of both pro- and anti-inflammatory mediators (Cheng et al. 2024; Almonajjed et al. 2025; Mamieva et al. 2022). The results of a study by Edogawa et al. (Edogawa *et al.* 2020) demonstrated the role of fecal proteases in the increased intestinal barrier permeability and disruption of tight junction proteins (Aggeletopoulou and Triantos 2024; Edogawa et al. 2020). Increased proteolytic activity was especially noticeable among patients with PI-IBS and was to affect the severity of symptoms (Aggeletopoulou and Triantos 2024; Edogawa et al. 2020). Furthermore, GM has been demonstrated to play a pivotal role in mucus production, which in turn serves as a protective barrier between the epithelial cells and the intestinal lumen (Aggeletopoulou and Triantos 2024). The composition of the mucus layer is primarily influenced by bacteria such as *Ruminococcus* spp., *Bacteroides thetaiotaomicron* and *F. prausnitzii* (Aggeletopoulou and Triantos 2024; Almonajjed et al. 2025).

4.4. Gut microbiota-brain axis

The gut-brain axis is defined as a system of bidirectional communication between the gastrointestinal tract and the nervous system (both the central nervous system and the autonomic nervous system) involving neuronal, endocrine and immune pathways (Cheng et al. 2024; Shrestha et al. 2022; Baj et al. 2019). This interaction has been demonstrated to regulate gut motility and sensitivity, also in addition to modulating emotional and pain responses (Aggeletopoulou and Triantos 2024). It is hypothesised that this connection is involved in IBS development. The concept of a GBA has been proposed, suggesting a potential role for GM in this process (Cheng et al. 2024). The GM has been implicated in the production of neurotransmitters (e.g. serotonin), modulators, and metabolites (e.g. short-chain fatty acids, tryptophan), as well as in maintaining the integrity of the intestinal barrier (Aggeletopoulou and Triantos 2024; Shrestha et al. 2022). The influence of the gut microbiome on the functioning of patients diagnosed with IBS is a subject of much debate, with studies suggesting both positive and negative influences (Aggeletopoulou and Triantos 2024). It has been observed that pathogenic bacteria, such as *Pseudomonas aeruginosa* and *Campylobacter jejuni*, have been shown to proliferate in an environment stimulated by stress-related neurotransmitters. These bacteria have been implicated in the enhancement of gut permeability, the onset of visceral pain, and, in the case of *P. aeruginosa*, the promotion of inflammatory activity (Aggeletopoulou and Triantos 2024). Cytokines, defined as proteins that regulate the immune system, have been implicated in inflammatory processes. The principal cytokines involved are IL-6, IL-8 and TNF-α, which have also been associated with stress, anxiety and depression in IBS (Almonajjed et al. 2025). Recent studies have demonstrated that neuroinflammation can be triggered by SCFAs produced by bacteria. These SCFAs have been observed to stimulate the recruitment of immune cells within the affected area (Shrestha et al. 2022). Conversely, beneficial microorganisms, exemplified by bacteria such as *Bifidobacterium* spp., have been observed to produce neurotransmitters including gamma-aminobutyric acid (GABA) and serotonin. The efficacy of these compounds in enhancing serotonin receptor expression and mitigating the deleterious effects of diverse stimuli on the brain has been demonstrated (Aggeletopoulou and Triantos 2024). Consequently, they have been shown to modulate patient mood and stress responses in a positive manner, thereby enhancing overall well-being

indicating considerable corpus of evidence has been amassed which indicates the involvement of GM in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. The process is primarily driven by pro-inflammatory cytokines (IL-6 and IL-8), which are produced by *Aspergillus fumigatus*, *Candida albicans*, and *Saccharomyces cerevisiae* fungi in the intestinal mucosa (Shrestha et al. 2022; Chong et al. 2019). The ultimate outcome of this axis is the secretion of cortisol from the adrenal cortex. Dysregulation of this axis is hypothesised to be the underlying cause of the psychological disorders experienced by patients with IBS, including anxiety, stress and depression. These disorders have been shown to affect visceral hypersensitivity, intestinal motility and permeability, GM composition and immune response (Mamieva et al. 2022; Chong et al. 2019). Among these patients, an increased abundance of *E. coli*, *Pseudomonas* spp., *Enterobacteriaceae* family, *Streptococcus* spp., *Prevotella* spp., and *Clostridium* spp. has been observed, while levels of *Lactobacillus* spp. have been shown to be decreased (Shrestha et al. 2022).

5. Changes in the gut microbiota composition in dependence on subtype of IBS

Recent research (Cheng et al. 2024; Chong et al. 2019; Surdea-Blaga et al. 2024) has focused on alterations in the qualitative and quantitative composition of the gastrointestinal microbiota in patients diagnosed with IBS, categorised by age and other health parameters (**Table 2**).

Table 2: Differences in gut microbiota qualitative-quantitative composition in depending on subtype of irritable bowel syndrome.

Subtype	Changes in gut microbiota		Ref.
	increase	decrease	
IBS-D	*Enterobacteriaceae*, *Proteobacteria*, *Firmicutes*, *Clostridiales*, *Bacteroides*, *Lactobacillus*, *Prevotella*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Dorea*	*Actinobacteria*, *Bacteroidetes*, *Ruminococcaceae*, *Methanobacteriaceae*, *Parasuterella*, *Lactobacillus*, *Bifidobacterium*, *Prevotella*, *Enterococcus*, *Faecalibacterium*, *Lachnospira*, *Turicibacter*,*Weisella*, *Oxolobacter*, *Oceanobacillus*, *Collinsella aerofaciens*	Cheng et al. 2024; Chong et al. 2019; Surdea-Blaga et al. 2024
IBS-C	*Bacteroides*, *Clostridiales*, *Christensenellaceae*, *Veilonella*, *Akkermansia*, *Methanobrevibacter*, *Pseudomonas aeruginosa*, *Methanobrevibacter smithii*	*Bacteroides*, *Methanobrevibacter*, *Bifidobacterium catenulatum*, *Prevotella*	Cheng et al. 2024; Chong et al. 2019; Surdea-Blaga et al. 2024
IBS-M	-	*Faecalibacterium prausnitzii*	Cheng et al. 2024
IBS-U	*Pseudomonas aeruginosa*	-	

6. Therapeutic approach in irritable bowel syndrome

A considerable number of therapeutic strategies have been developed for the purpose of modulating the composition of the GM in patients diagnosed with IBS. These strategies encompass dietary modifications, the supplementation of antibiotics, probiotics, synbiotics, prebiotics, postbiotics, and FMT (Cheng et al. 2024; Aggeletopoulou and Triantos 2024).

6.1. Diet

In the context of treating patients suffering from IBS, diet plays a pivotal role, with low FODMAPs being of particular significance. Evidence suggests that this ddietary is efficacious in reducing symptoms associated with IBS, including bloating, visceral pain and general discomfort (Cheng et al. 2024; Almonajjed et al. 2025; Chong et al. 2019). The ingestion of plant-based proteins has been associated with an increased levels of beneficial bacteria (e.g. *Bifidobacterium* spp., *Lactobacillus* spp.) and a decreased levels of pathogenic bacteria (e.g. *Bacteroides fragilis* and *Clostridium perfringens*) (Shaikh et al. 2023). The low FODMAP diet has also been observed to reduce inflammatory activity and increase gut permeability (Aggeletopoulou and Triantos 2024). However, it is important to note that this dietary approach is associated with certain disadvantages, including nutritional deficiencies, reduced fibre intake, constipation, and an imbalance in GM composition, characterised by decreased levels of beneficial bacteria (Cheng et al. 2024; Almonajjed et al. 2025). Additionally, the efficacy of this therapeutic approach may be subject to variation depending on the IBS subtype (Almonajjed et al. 2025).

6.2. Antibiotics

Antibiotics have been posited as a potential novel therapeutic approach in the management of IBS. In clinical practice, a range of antibiotic medications have been employed, including neomycin, doxycycline, amoxicillin/clavulanate, norfloxacin, and rifaximin (Cheng et al. 2024; Shaikh et al. 2023). Notably, the lat-

ter was endorsed by the American Journal of Gastroenterology for the management of IBS (Shaikh et al. 2023). The benefits of rifaximin include a limited spectrum of side effects, low levels of resistance and toxicity, and ease of administration (by mouth) (Shaikh et al. 2023; Chong et al. 2019). The effectiveness of rifaximin was emphasized in two clinical trials (TARGET 1 and TARGET 2), where improvements in symptoms were evident among patients with IBS-D in comparison to the control group (Shaikh et al. 2023). Antibiotics, as a form of targeted therapy, have been shown to reduce levels of pathogenic bacteria, such as *E. coli* and *Enterobacteriaceae* (Aggeletopoulou and Triantos 2024). The beneficial effect of antibiotics in IBS has been observed in the reduction of symptoms, including bloating or general discomfort (particularly in IBS-D), and alterations in immune and inflammatory responses (Aggeletopoulou and Triantos et al. 2024; Shaikh et al. 2023). It is imperative to emphasise that patients diagnosed with IBS should adhere to antibiotic usage guidelines, as misuse of these medications can lead to an escalation in bacterial resistance, the emergence of adverse effects, resistance to the antibiotic treatment, and a disruption in the intestinal microbiota (i.e. dysbiosis) (Cheng et al. 2024).

6.3. Prebiotics, probiotics, synbiotics and postbiotics

The qualitative and quantitative composition of the GM can be modified to a considerable extent through simple means (**Table 3**). Such modifications can be achieved by adjusting dietary habits to incorporate fibre-rich foods, as well as by introducing probiotic bacterial supplements that have been demonstrated to possess beneficial properties. The efficacy of a probiotically enriched microbiome can be augmented by paraprobiotic preparations (i.e. non-viable, inactivated bacteria or their cellular components) and/or postbiotic preparations (i.e., products of bacterial metabolism or equivalent synthetic products that beneficially modulate the immune response of the macroorganism and reduce inflammation) (Martyniak et al. 2021). The aforementioned approaches are used to: (a) the binding of immune function, (b) the alleviation of symptoms of irritable bowel disease, (c) the reduction of the severity of allergies, (c) the prevention and treatment of tooth decay, and (d) the prevention and treatment of metabolic syndrome (Luzzi et al. 2024).

Table 3: Prebiotics, probiotics, synbiotics and postbiotics used in alleviating symptoms of irritable bowel syndrome

Biotics in prophilaxis or therapeutic approach	Potential use for the prevention and treatment	Therapeutic activity	Ref.
Probiotics	Beneficial bacteria strains that can be administered orally as a dietary supplement	The reduction on gut inflammation, increase the level of beneficial bacteria (*Lactobacillus* spp., *Bifidobacterium* spp.), inhibition of growth of pathogenic bacteria, modulation of both anti- and pro-inflammatory cytokines, participation in production of SCFAs, production of neurotransmitters, improve symptoms in IBS (e.g. abdominal pain, bloating), tighten gut barrier, regulation of GBA, improve gut barrier integrity and mucus production, reduction of intestinal permeability, improve patient's quality of life and mood, influence on the both innate and adaptive immunity, with interaction occurring with epithelial cells, dendritic cells, macrophages and lymphocytes through pattern-recognition receptors, helping regulate Tcell balance (especially boosting Treg. to reduce inflammation), prevention antibiotica-ssociated diarrhea, necrotizing enterocolitis, pouchitis, and traveler's diarrhea, *in vitro* and animal studies indicate improved burn wound healing with *Saccharomyces cerevisiae*, and prevention or reduction of eczema through mechanisms involving the GBA	Cheng et al. 2024; Aggeletopoulou and Triantos 2024; Almonajjed et al. 2025; Shaikh et al. 2023; Martyniak et al. 2021; Luzzi et al. 2024; Qiao et al. 2025; Maftei et al. 2023; Campaniello et al. 2023; Rijkers et al. 2011; Ranjha et al. 2021; Fuochi and Furneri 2023

Prebiotics	• Dietary fibers that are non-digestible food components by human enzymes and not absorbed by the human small intestine. They reach the colon where they are fermented by bacteria present in the GM • Present naturally in multitude of plant foods, including artichokes, asparagus, chicory, garlic, onions, wheat, and bananas • It can be synthesised and incorporated into food products	Promotion of growth of beneficial bacteria, improve symptoms in irritable bowel syndrome, production of SCFAs (including byturate, propionate and acetate), regulation of gut motility, improve intestinal barrier function, reduction of inflammatory processess, anti-oxidative activity, regulation of cholesterol and lipids synthesis	Aggeletopoulou and Triantos 2024; Almonajjed et al. 2025; Shaikh et al. 2023; Chong et al. 2019; Martyniak et al. 2021; Luzzi et al. 2024
Synbiotics	• Products that contain both prebiotics and probiotics • It is possible to formulate such products in two different ways: the first approach, known as the complementary approach, the prebiotic and probiotic substances work independently; in the second approach, known as the synergistic approach, the prebiotic and probiotic substances work together	Improve probiotics survival in gastrointestinal tract, reduction of symptoms in IBS (bloating, abdominal pain), increase a bowel movement frequency, reduction of levels of pro-inflammatory cytokine (IL-8, TNF-α) and increase of levels of anti-inflammatory cytokines (IL-10), improve intestinal barrier integrity and gut motility	Almonajjed et al. 2025; Shaikh et al. 2023; Chong et al. 2019; Martyniak et al. 2021; Luzzi et al. 2024
Postbiotics	• Classified as either (a) products resulting from bacterial metabolism, or (b) synthetic products that possess the capability to modulate inflammation and the immune response	It is assumed that improve symptoms in IBS (particularly in IBS-D) and reduce inflammatory activity	Almonajjed et al. 2025; Martyniak et al. 2021

6.4. Fecal microbiota transplantation (FMT)

Another therapeutic approach in the treatment of IBS is FMT. This strategy involves the transfer of a stool solution from healthy individuals to patients with IBS, with the objective of restoring a healthy GM composition, improving its diversity, increasing the level of beneficial bacteria and decreasing the level of pathogenic species particularly associated with IBS (Almonajjed et al. 2025). FMT leads to strengthening of the intestinal barrier, reduction of inflammatory processes, modification of the immunological response and, potentially, improvement of the GBA (Almonajjed et al. 2025). The application of FMT in the treatment of patients infected with *Clostridioides difficile* has been documented (Cheng et al. 2024; Shaikh et al. 2023). FMT donors might be both healthy relatives or anonymous. In case of anonymous donors there is an oppor-

tunity to select donors with a high diversity in the composition of GM and obtained stool might be stored in the freezers by a long time and then used for multiple patients (Cammarota et al. 2019; Halkjær et al. 2023). There are several methods which can be used in FMT including endoscopic procedures or using gastro-duodenal or rectal tube. Also capsules delivery led to release the stool in the small intestines (Halkjær et al. 2023). The results obtained from various randomised clinical trials (RCTs) have been found to be inconsistent. Studies carried out by El Salhy et al. (El-Salhy et al. 2019), Johnsen et al. (Johnsen et al. 2018) and Holvoet et al. (Holvoet et al. 2021) have demonstrated a favourable clinical response following FMT treatment, characterized by an enhancement in symptoms related to IBS, in comparison to the control group that received a placebo. Conversely, the results of randomised clinical trials conducted by Halkjær et al. (Halkjær et al. 2018) demonstrated that the control group (placebo) exhibited a superior clinical response in comparison to patients who had undergone FMT. The observed variations in outcomes among studies may be attributable to several factors, including individual patient characteristics, delivery method, or donor selection (Almonajjed et al. 2025). At present, FMT is not recommended as a first-line treatment for IBS, and further research is required to ascertain the beneficial effect of FMT on the therapeutic success of patients with IBS (Cheng et al. 2024; Chong et al. 2019).

6.5 Mind-body therapies

The experiences of numerous clinicians have underscored the necessity to monitor the mental health of patients diagnosed with IBS. In the context of diagnosis and treatment, the incorporation of patient surveys has been demonstrated to facilitate the delivery of holistic care, encompassing a combination of medication, dietary consultations, and psychological support. The significance of educating patients with psychosomatic disorders in the ability to name and recognise emotions and cope with stress is also emphasised. In cases where patients are experiencing symptoms that are deteriorating as a result of anxiety or stress, the utilisation of mind-body therapies, cognitive-behavioral therapies (including hypnosis, meditation, various forms of relaxation or biofeedback), is recommended (Chey et al. 2020). The International Foundation for Gastrointestinal Disorders also recommends diaphragmatic/abdominal breathing techniques, progressive muscle relaxation by tensing and then relaxing different muscle groups, and visualisation/positive imagery techniques to facilitate the imaginative process of envisioning oneself in a calm, quiet and relaxing place. By focusing on a particular place, the patient is able to divert their attention away from disturbing thoughts. It is imperative that patients with IBS invest time in acquiring knowledge about the condition, identifying potential triggers for symptoms, and engaging in the relaxation exercises that have been outlined. This approach enables them to take proactive, constructive, and innovative measures to enhance their ability to cope with and manage their symptoms effectively (Zeichner 2005).

It is imperative to adopt effective coping mechanisms to manage the stress and anxiety that may be precipitated by IBS. It has been demonstrated that breathing exercises, meditation and yoga can assist in the reduction of stress and tension. Relaxation techniques can be used in two ways: as a supplement to pharmacological therapy or as an alternative when medication is not sufficiently effective (Chey et al. 2020; Zeichner 2005).

7. **Limitations of current therapies**

It is worth highlighting that the therapeutic options currently employed in the treatment of IBS are associated with several limitations. There is a paucity of long-term data on the effects of probiotics as a therapeutic treatment and the adverse events associated with it. It is imperative to acknowledge that the efficacy of probiotic therapy is contingent upon the specific strain used, the dosage administered and the duration of the treatment regimen. To date, these factors have not been optimised for IBS subtypes. The safety profile is also not unclear, particularly in the case of long-term therapy (Umeano et al. 2024; Almonajjed et al. 2025). A further limitation of the IBS therapies in current use is the relatively small sample size and the limited duration of the studies. This limits the capacity to derive robust conclusions, particularly with regard to the efficacy of probiotics in managing different subtypes of IBS. Further research is required with larger populations and longer durations in order to evaluate the long-term efficacy of probiotics and to determine their effect on different subtypes of IBS (Ruiz-Sánchez et al. 2024; Almonajjed et al. 2025). A considerable number of medications, comprising antispasmodics,

antidepressants and several novel agents, have been observed to offer only a marginal improvement in IBS symptoms. It is important to note that the alleviation of gastrointestinal symptoms does not necessarily result in a substantial enhancement of the patient's overall quality of life. This underscores the necessity for a holistic approach (Talley 2003; Sainsbury and Ford et al. 2011; Hammerle et al. 2008; Brenner et al. 2024). Certain medications, notably older antidepressants such as tricyclic antidepressants (TCAs), have been observe to induce significant adverse effects that can potentially restrict their utilisation, especially in individuals suffering from IBS, who may already be afflicted by gastrointestinal discomfort (Wall et al. 2011; Lacy et al. 2009). Despite the evidence that brain-gut behaviour therapy (BGBT) is efficacious in the amelioration IBS symptoms and quality of life, access to this therapy is limited by a paucity of trained practitioners, patient time constraints and cost. Furthermore, clinicians may also have a lack of awareness of the specific nature of BGBT and its distinction from general psychotherapy, which may potentially hinder referrals (Brenner et al. 2024). Personalised treatment strategies that consider individual symptom profiles, dietary factors, and psychological aspects are often required, but their implementation can be complex (Sainsbury and Ford et al. 2011).

8. Future perspectives

The integration of advanced omics technologies and machine learning techniques has the potiential to significantly enhance future research in then compassing the analysis of microbiome composition and the identification of therapeutic targets. Combined with next-generation sequencing (NGS) technologies, such as shotgun metagenomics, provide deeper insights into the structure and function of the GM. The application of advanced techniques (metatranscriptomics or metabolomics) holds promise in enhancing our comprehension of the microbial functional pathways that contribute to the pathogenesis of IBS. These methods provide a more detailed picture of the complex interactions between the microbiome and host physiology, helping to identify novel therapeutic targets necessary to develop effective microbiome-targeted interventions. The use of these instruments has the capacity to expedite the identification of diagnostic biomarkers, improve patient risk assessment, and refine the prediction of treatment response in IBS (Fukui et al. 2020; Jacobs and Lagishetty et al. 2023; Aggeletopoulou and Triantos 2024). Current research on IBS is confronted with a number of methodological challenges. The majority of studies rely on the sequencing of the 16S ribosomal RNA subunit (rRNA) gene, which offers only genus-level resolution and fails to provide functional insight into the microbiome. In contradistinction to NGS, more advanced techniques, such as shotgun metagenomic sequencing and RNA sequencing, offer greater sensitivity, resolution and deeper understanding of microbial structure and function. Furthermore, most studies focus on stool samples, which may not fully represent the microbiome of other intestinal regions, such as the small intestine or mucosal layer. In addition, while some studies assess the microbiota at different time points, most are limited to two measurements, making it difficult to track changes in microbiota and metabolites over time, especially during disease exacerbations or remission (Aggeletopoulou and Triantos 2024; Ankersen and Weimers 2021; Ek and Reznichenko 2015; Mars and Yang 2020; Meydan and Afshinnekoo 2020).

9. Conclusions

Irritable bowel syndrome is a multifactorial gastrointestinal disorder involving numerous factors, including genetic predisposition, psychoenvironmental factors, and alterations in the composition of GM. Scientific reports published in recent years indicate that GM play a crucial role in the development and progression of IBS, particularly in cases of reduced levels of certain GM species, a condition referred to as dysbiosis. The involvement of GM in numerous processes associated with IBS has been well-documented, including nutrient absorption in the intestines, immune response regulation, the functioning of the GBA, and mucosal immune regulation. However, due to the substantial interindividual variability, it remains challenging to identify a universal GM composition in IBS. The efficacy of the available treatment methods is a contentious issue. The therapeutic approach to IBS should be personalised, and future research should focus on the search for microbial species that might be used as biomarkers for IBS. These biomarkers would help to differentiate between the subtypes of this gastrointestinal disorder.

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UNDERSTANDING PROBIOTICS, PREBIOTICS, SYNBIOTICS, AND POSTBIOTICS: A COMPREHENSIVE REVIEW OF THE NEWEST DEFINITIONS, SELECTED STRAINS AND PRODUCTS

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Abstract: In recent years the field of probiotics, prebiotics, synbiotics and postbiotics has been extensively studied. Preparations including live and inanimate microorganisms, their parts and substances that selectively stimulate their growth, are promising in treatment or amelioration of symptoms in many diseases. The aftermath of the COVID-19 pandemic has forced us to face complications such as post-acute COVID-19 syndrome and a general decrease in population immunity, for which treatment with probiotics, prebiotics, synbiotics and postbiotics is promising. The use of such preparations can have a positive effect on the immune system and has also shown positive effects in major depressive disorder. Due to the rapid development in the field a lot of confusion and misconceptions emerged, especially regarding the use of terms and definitions. This article aims to present a clear classification of these products according to International Scientific Association of Probiotics and Prebiotics (ISAPP) guidelines as well as basic mechanisms of action and efficacy of selected preparations. Authors of this article use the term 'biotic(s)' to refer collectively to probiotics, prebiotics, synbiotics and postbiotics. While this term has not been officially established, it is used by other authors in the scientific literature. The taxonomic nomenclature used in this article has been updated according to the most recent taxonomic reclassification.

1. Introduction. 2. Current classification and nomenclature for biotics. 2.1. Probiotics 2.2. Prebiotics. 2.3. Synbiotics. 2.4. Postbiotics. 3. Navigating synonyms: challenges in biotics nomenclature. 3.1. Biotics complementary mode of action and health benefits. 3.2. Molecular pathways. 3.3. Single vs multiple-strain probiotics. 3.4. Efficacy and regulatory framework of biotics. 3.5. Future perspectives.

Keywords: probiotics, prebiotics, synbiotics, postbiotics, next-generation probiotics, live biotherapeutic products;

1. Introduction

The practice of using fermentation in food preparation and preservation dates back far in the history of human species across the globe. Despite the widespread use, it was no earlier than the beginning of 20th century that Metchnikoff proposed the beneficial value of consuming fermented foods and associated these benefits with lactic acid bacteria (LAB) (Metchnikoff, 1907; Markowiak and Śliżewska, 2017). Since then, the idea of beneficial influence of bacteria on humans has been extensively investigated by scientists, ultimately leading to the formulation of the term 'probiotic' in 1954 and its first definition in 1965 (Vergin, 1954; Lilly and Stillwell, 1965). The development in the field of probiotics, which also led to formulation of new definitions for other biotics, has created some misconceptions regarding the understanding and proper use of these terms.

The field of biotics is characterized by a variety of terms that frequently denote the same idea. Although the concept of probiotics is widely understood and ac-

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cepted, other biotics such as synbiotics or postbiotics encounter challenges due to the lack of clear understanding and the presence of synonymous terms and definitions. Therefore, the International Scientific Association for Probiotics and Prebiotics (ISAPP) was founded to bring together expert scientists in the field. ISAPP proposed four terms and their definitions in 2014, 2017, 2020 and 2021 to create a unified nomenclature, respectively: probiotic, prebiotic, synbiotic and postbiotic (Table I). The establishment of each term, definition, and clear guidelines was preceded by a convention of a panel of experts. Most of the authors in the field assert that the nomenclature and definitions provided by ISAPP most accurately describe all microorganism-derived products and substrates that are selectively utilized by microorganisms, conferring a health benefit (Hill et al. 2014; Gibson et al. 2017; Swanson et al. 2020; Salminen et al. 2021). In this paper the authors aim to compile the most current definitions of all biotics according to ISAPP recommendations and present them clearly, highlighting the differences and connections. Additionally, authors discuss modes of action of biotics and characterize selected probiotic strains.

2. Current classification and nomenclature for biotics

2.1. Probiotics

The term "probiotic" was first used by Vergin in 1954 in the paper "Anti-und Probiotika" (Vergin, 1954). Lilly and Stilwell presented first definition, describing probiotics as a growth-promoting factors produced by one microorganism that exert beneficial effects on another microorganism (Lilly and Stillwell, 1965). The most recent definition was proposed by the FAO/WHO in 2001 and was accepted, with minor grammatical change, by ISAPP in 2014 as: "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001; Hill et al., 2014). This definition is clear and rarely misused. The ISAPP has published clear guidelines that precisely define whether the definition is applicable – Table I and Figure I (Hill et al., 2014).

One of the first described probiotic strains was *Lactobacillus bulgaricus*, isolated by Grigorov in 1905 (Lee et al. 2024). In 1985 Gorbach and Goldin isolated and described *Lactobacillus rhamnosus* GG (for more probiotic strains and their health benefits see Table II). Following the reclassification of the *Lactobacillus* genus, this strain was renamed to *Lacticaseibacillus rhamnosus* GG (Stage et al. 2020; Zheng et al. 2020).

When defining a probiotic one should determine whether beneficial effects are species-specific or strain-specific. This association can be defined in respect of the claims for a certain probiotic. If the claims exceed core benefits, then the probiotic should be defined at strain level. Core benefits allow for generalization of certain effects or mode of action present at species level. Examples of such benefits include colonization resistance, short-chain fatty acids (SCFA) production, vitamins synthesis or direct antagonism (Beane et al., 2021; O'riordan et al., 2022; Zhang et al., 2022; Caballero-Flores et al., 2023). For more distinct effects such as neurological, endocrinological and immunological effects, a strain-specific relation should be applied accordingly (Hill et al., 2014).

Table I. Current classification and nomenclature of biotics according to ISAPP.

Name	ISAPP definition	Examples	Examples of commercial products	Reference
Probiotic	Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.	*Lactobacillus acidophilus* DSM 20079, *Lactiplantibacillus plantarum* 299v, *Bifidobacterium longum* subsp. *infantis* UCD272, *Saccharomyces boulard*ii CNCM I-745	Vivomixx® Lacidofil® Enterol®	Hill et al., 2014
Prebiotic	A substrate that is selectively utilized by host microorganisms conferring a health benefit.	galactooligosaccharides (GOS), fructooligosaccharides (FOS), Inulin	Orafti® Inulin NutraFlora® FOS BLF®100	Gibson et al., 2017

Synbiotic	A mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host.	*Lactiplantibacillus plantarum* ATCC 202195 and fructooligosaccharides (FOS)	Ther-Biotic® Synbiotic Acidolac® Baby Multilac®	Swanson et al., 2020; Kleerebezem and Führen, 2024
Postbiotic	Preparation of inanimate microorganisms and/or their components that confers a health benefit on the host.	pasteurized *Akkermansia muciniphila* Muc^T heat-killed *Lacticaseibacillus paracasei* MCC1849	SANPROBI® Premium EpiCor® BPL1™ Postbiotic	Salminen et al., 2021; Kato et al., 2024

The strain-specific effects of a probiotics can also extend to mental health benefits leading to the formulation of a term 'psychobiotic'. Psychobiotics are promising therapeutics for diseases such as schizophrenia, depression, autism spectrum disorder, Alzheimer's disease, Parkinson's disease, or Tourette syndrome (Logan and Katzman, 2005; Liu et al., 2019; Munawar et al., 2021; Sharma et al., 2021). Examples of psychobiotics include *Lactiplantibacillus plantarum* PS128 which has been used to ameliorate some autism symptoms (Liu et al. 2019); four probiotic strains (*Bifidobacterium infantis* Bi-26, *Lacticaseibacillus rhamnosus* HN001, *Bifidobacterium lactis* BL-04, and *Lacticaseibacillus paracasei* LPC-37) administered together with FOS which positively affected the children with autism spectrum disorder (ASD), contributing to behavioural and gastrointestinal (GI) tract improvement (Wang et al. 2020); and *Bifidobacterium breve* CCFM1025 which attenuates psychiatric and gastrointestinal abnormalities in patients with major depression disorder (Tian et al. 2022).

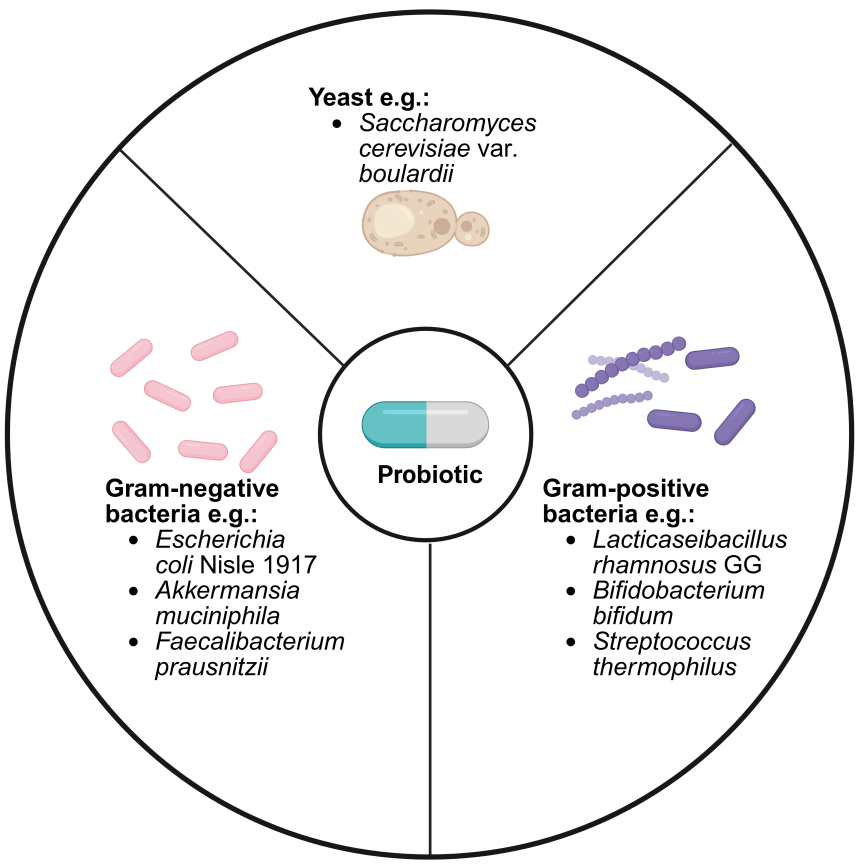

Figure 1. Classification of microorganisms constituting probiotics with representative examples. Created in BioRender.

Classifying psychobiotics as a separate group contradicts the goal of unifying and simplifying scientific nomenclature. There is no unified definition of psychobiotic, but most authors describe psychobiotics as probiotics with the specific characteristic that their claimed health benefits are associated with mental health (Magalhães-Guedes, 2022; Zhu et al., 2023; Chiano et al., 2024). Based on this common understanding, they should be identified as a specific type of probiotic or sub-group/sub-class rather than a separate group of biotics. Some authors expand the definition of psychobiotic to include "any exogenous influence whose effect on the brain is bacterially mediated" encompassing prebiotics as well (Sarkar et al., 2016, 2020; Warda et al., 2019). The authors of this paper disagree with such an approach, as it broadens the concept of psychobiotic to include any biotic or any substance beyond the field of biotics. This approach makes it unclear as to what a psychobiotic might be composed of, allowing for the possibility that two entirely different preparations could share the same name. Psychobiotics should be understood as "probiotic bacteria that benefit mental health when consumed in adequate amounts" (Dziedzic et al. 2024).

An important aspect of probiotics is the incorporation of genetically modified microorganisms (GMMs) (Ma et al. 2022). Each strain's safety must be assessed regardless of the modification (Zhou et al. 2020). Genetic engineering and tools such as CRISPR/Cas9 facilitate the development of GMMs (Wu et al. 2021; Chen et al. 2025). ZBiotics® is one of the few probiotics based on GMMs and the first to become commercially available. It was designed to ameliorate the hangover symptoms, using *Bacillus subtilis* modified with the acetaldehyde dehydrogenase gene derived from *Cupriavidus necator* (Esawie et al. 2025). This probiotic also has potential for addressing type 2 diabetes mellitus and non-alcoholic steatohepatitis (Saad et al. 2024; Esawie et al. 2025). It has been proposed that GMMs should be excluded from probiotics, with next-generation probiotics (NGP) and live biotherapeutic products (LBP) taking on that role (O'Toole et al. 2017).

Warda et al. proposed that the definition of probiotics should also include inactivated microorganisms (Warda et al., 2019). Inanimate bacterial cells fall under the definition of postbiotic and used to be referred to as 'heat-killed probiotics', 'paraprobiotics' and other synonymous names, prior to the consensus statement on the definition of postbiotics (Salminen et al., 2021). Nevertheless, creating a new classification that includes components for which definitions have already been coined and for which clear classification have been established, is unnecessary and hinders the development in the field of biotics. All microorganisms, their products and substrates for selective utilization can be described using four basic and defined terms (probiotic, prebiotic, synbiotic and postbiotic) or the appropriate chemical name of isolated metabolite. Thus, creating a new definition seems unnecessary (Hill et al. 2014; Gibson et al. 2017; Swanson et al. 2020; Salminen et al. 2021).

2.2. Prebiotics

The concept of prebiotics was introduced in 1995 by Gibson and Roberfroid. Initially the following definition was proposed "A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health" (Gibson and Roberfroid, 1995). In this initial understanding prebiotics were exclusively connected with GI tract, as they only referred to food ingredients. Further development of the concept led to the formulation of a new definition: "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). This change broadened the idea of prebiotics, allowing for substances other than carbohydrates, which do not have to be present in food and can be applied to body sites other than GI tract, to be classified as prebiotics.

An important aspect of a prebiotics is their selectivity, which was highlighted in the initial definition and persists in the most recent understanding of the term. Selectivity differentiates the prebiotics from dietary fibre and other substances that affect the microbiota in non-selective manner. While the dietary fibre is not digested by the host, sharing this characteristic with prebiotics, it can be utilized by gut microbiota in general. Prebiotics, however, are utilized only by given

group or groups of microorganisms, which, along with the health benefit, ought to be proven experimentally (Hutkins et al. 2024). The beneficial aspects of prebiotics include increased abundance of beneficial microbiota e.g. *Bifidobacterium spp*. which produce metabolites such as SCFA (Lai et al. 2023). The effect does not have to be direct as long as the health benefit is obtained. An example of this is the 'cross-feeding effect', where the production of a beneficial product, positively affecting host health, results of interaction between two microorganisms induced by a prebiotic (Culp and Goodman 2023). Such interaction has been observed between *Bifidobacterium longum* PT4 and *Bacteroides ovatus* HM222. When xylan was used as a carbon source, the *B. longum* PT4 showed an increased growth in the presence of *B. ovatus* HM222, indicating potential cross-feeding effect (Vega-Sagardía et al. 2023).

The most common prebiotics are galactooligosaccharides (GOS), fructooligosaccharides (FOS) or inulin (Flaujac Lafontaine et al. 2020). Candidates for prebiotics are constantly being researched, with human milk oligosaccharides (HMO) being an example. Human milk oligosaccharides play an important role in early stages of gut microbiota development. HMO are selectively metabolized by *Bifidobacteriaceae* and especially *Bifidobacterium longum* subsp. *infantis*. They can also prevent pathogen adhesion, making HMO very promising candidates for prebiotic (Okburan and Kızıler, 2023).

To conclude, the most important characteristic of prebiotics are: being non-digestible by host, selectively stimulating the growth and/or the activity of a group of microorganisms, conferring health benefit to the host (Jenkins and Mason 2022).

2.3. Synbiotics

The concept of synbiotics emerged alongside prebiotics. It was the same article where Gibson and Roberfroid defined prebiotics, they also proposed the concept of the synbiotics as the combination of probiotics and prebiotics (Gibson and Roberfroid 1995). The initial definition described synbiotics as: "a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria and thus improving host welfare" (Gibson and Roberfroid, 1995).

The definition was updated by ISAPP in 2020, describing synbiotics as: "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" (Swanson et al., 2020). The updated and simplified definition broadens the understanding of the term. Combination of prebiotics and probiotics are still referred to as synbiotics, specifically as complementary synbiotics - Figure 2. Such products are not designed to work exclusively together, they are administered together but each component must be a defined biotic separately (with all the requirement for each accordingly). The effect of complementary synbiotic is no greater than when the components of the synbiotic are administered separately. Updating the definition allowed for the concept of a synergistic synbiotics to emerge. Elements of such synbiotics do not have to be a predefined prebiotics and probiotics. The microorganism and the substance used in the formulation depend on one another in such way that, when used separately, they exert much weaker or no health benefit. Such approach allows for a development of new synbiotics, components of which haven not necessarily been used previously in other biotics. It is also important to note that in the most recent definition of synbiotics, the understanding of host microorganism both refers to autochthonous and allochthonous microbiota, latter administered in synbiotics or probiotics (Swanson et al., 2020). This is crucial since microorganisms present in synergistic synbiotic formulations might lack the ability to colonize the gut (Walter et al. 2018). Most commercially available synbiotics are complementary (Gomez Quintero et al. 2022). To the best of authors' knowledge, no synergistic synbiotic formulations are currently available on the market. However, *ex vivo* studies have demonstrated the potential of synergistic synbiotics, highlighting the need for further research, particularly through *in vivo* investigations (De Bruyn et al., 2024; Ghyselinck et al., 2024).

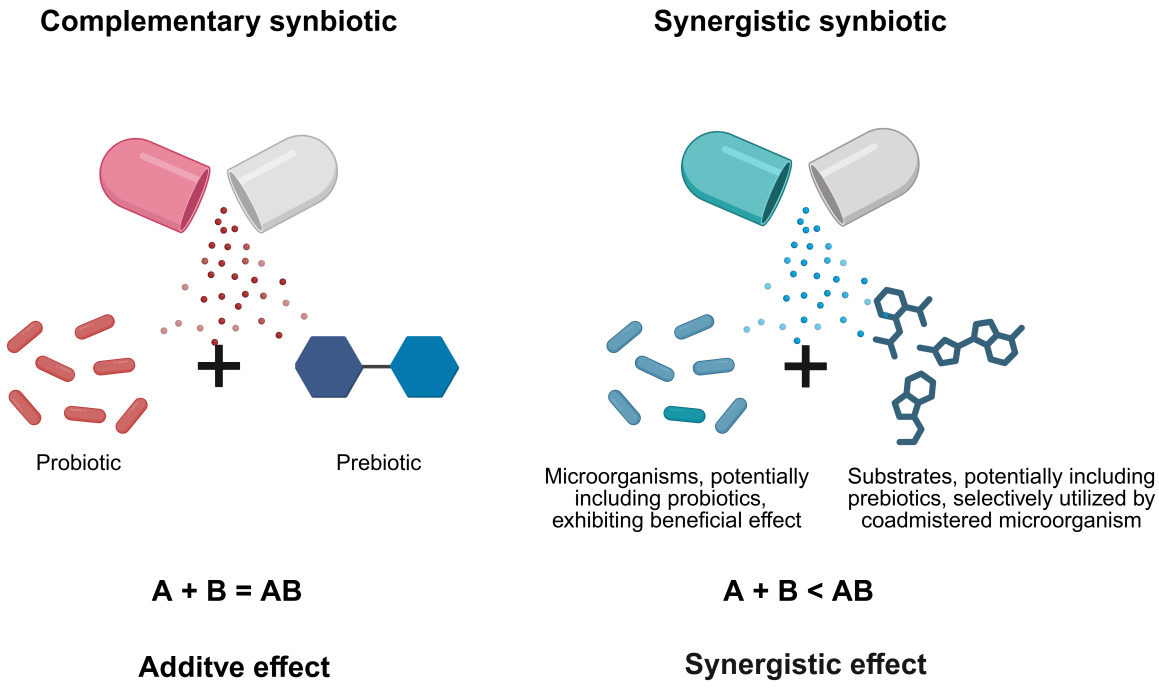

Figure 2. Comparative characteristics of complementary and synergistic synbiotics. Created in BioRender.

2.4. Postbiotics

Probiotics, in addition to mandatory presence of live microorganism, naturally contain dead cells. For a long time, the influence of dead microorganisms in probiotics has been overlooked. Since the potential of inanimate cells to confer a health benefit in host has been recognized, multiple names to describe such preparations have emerged in the literature. Examples include: 'heat-killed probiotics', 'paraprobiotics', 'tyndallized probiotics' and 'postbiotics' (Barros et al. 2021; Ding et al. 2021; Boyte et al. 2023; Bolzon et al. 2024). In 2019, ISAPP reviewed existing names describing preparations containing dead microorganism cells and, two years later, published the consensus statement on the definition of postbiotics: "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021).

The term "postbiotic" is coherent with other defined biotics and well describes the characteristics of the preparation - Figure 3. It is important to distinguish vaccines, which can include dead microorganism cells, and purified metabolites of microorganisms from postbiotics. Vaccines and metabolites do not fall under the definition of postbiotic. Metabolites can be present in postbiotic preparations but only together with dead cells and/or their parts (Salminen et al., 2021). Microbial metabolites can be named according to their chemical structure or origin, thus creating additional definitions such as: "compounds produced by the microbial metabolism, namely postbiotics" seems unnecessary (Puccetti et al. 2020).

Postbiotics, unlike vaccines, do not aim to provide post-vaccination immunity (Aggarwal et al. 2022). While they can affect the immune system, their effects differ fundamentally from those induced by vaccines (Shukla and Shah 2018). Moreover, postbiotics are not designed to prevent any specific diseases, which is the primary purpose for vaccines. For these reasons, associating postbiotics with vaccines is both incorrect and misleading (Salva et al. 2021; Prygiel et al. 2022).

Even though the clear definition of postbiotics has been proposed, authors still use synonymic names, such as: paraprobiotics (Lee et al., 2023; Mudaliar et al., 2024), heat-killed probiotics (Poaty Ditengou et al., 2023; Yoon et al., 2024), tyndallized probiotics (Bolzon et al., 2024). These multiple terms often describe the same concept, yet some involve modified definitions. For instance, "paraprobiotics, which contain inactivated nonviable probiotics" (Docampo et al., 2024). This understanding narrows the potential of inanimate microorganisms that could be used in preparations, since they would have to be also identified as probiotics, which is not obligatory for postbiotic preparations.

This narrow understanding also excludes metabolites and cell parts, which are included in the broader postbiotic definition (Vinderola et al. 2022).

The term 'heat-killed probiotics', used for preparations containing dead microorganism cells that provide a health benefit on the host is also problematic. Probiotics, according to their well understood and widespread definition must contain: "live microorganisms that, when administered in adequate amounts" (Hill et al., 2014). Hence, the use of the name probiotic for microorganisms that have been heat-killed seems inappropriate. The inconsistent use of multiple names for the same definition is highly unfavourable and hampers the development of postbiotics (Vinderola et al. 2024).

As mentioned above, the microorganisms used in postbiotic preparations do not have to be classified as probiotics, though they have to be clearly defined. This is important in context of the safety of use such as the presence of genes conferring antibiotic resistance (Daniali et al. 2020). The method of inactivation is yet another important aspect of postbiotics. Different methods of inactivation may influence the cells in different ways, thereby altering the characteristics of the final product (Zhong et al., 2024). Inactivation methods can broadly be categorized into two groups: thermal and non-thermal (Zhu et al., 2025). The use of temperature, in methods such as sterilization, pasteurization, freeze drying or spray drying, remain the most used due to standardized procedures and relatively low operational costs (Rafique et al., 2023). However, these approaches have notable limitations, as they may compromise beneficial cellular properties during the inactivation process (Sun et al., 2023). Non-thermal inactivation methods include UV, ultrasonic sterilization, high-pressure, pulsed electric field, irradiation, supercritical carbon dioxide and exposure to extreme pH conditions (Zhu et al., 2025). Those physical and chemical methods allow heat-labile elements to retain their bioactivity (Zhong et al., 2022). The use of inanimate microorganisms may also enable researchers to use genetically modified organisms, as the safety of use when microorganisms are administered in non-viable form is superior (Salminen et al., 2021).

One of the challenges in development of postbiotic preparations is the evaluation of number of cells and/or their parts present in the preparation. Establishing the CFU by plating method is prone to undervaluation of the cells present, as this technique omits dead cells. Flow cytometry (FCM) seems to be more applicable, as it can differentiate live and dead cells (Bolzon et al., 2024).

Though postbiotics face challenges, they can be superior to probiotics. Probiotic shelf life is a problem due to the mandatory presence of live microorganisms at declared concentration. The use of dead cells in postbiotics eliminates the problem of CFU fluctuations during shelf life, proposing a good alternative (Salminen et al., 2021).

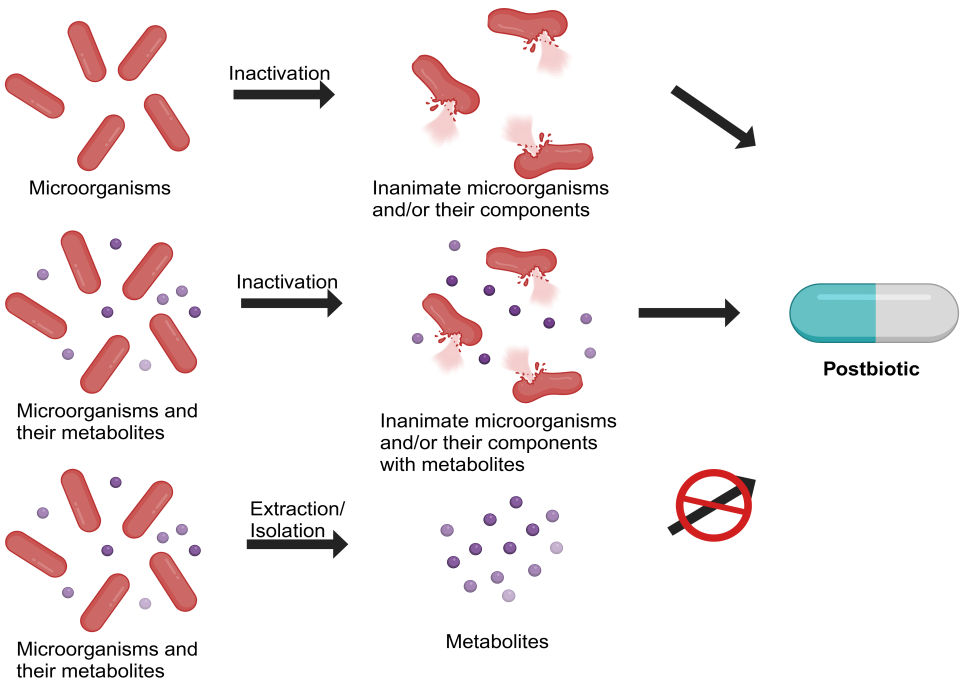

Figure 3. Postbiotic components vs. independent metabolites (Salminen et al. 2021; Vinderola et al. 2024). Created in BioRender.

3. Navigating synonyms: challenges in biotics nomenclature

The field of biotics is rife with synonymous terms and definitions, which hinder its development. Stakeholders may overlook significant literature related to the given topic due to the presence of multiple names, especially when they are not familiar with all the existing synonyms. The ISAPP has presented four names (probiotic, prebiotic, synbiotic and postbiotic), definitions and clear guidelines for each. Nonetheless, as highlighted above, incoherent nomenclature remains prevalent (Warda et al. 2019; Lee et al. 2023; Yoon et al. 2024).

There are three terms in the field that authors find particularly important to discuss: psychobiotic, next-generation probiotic (NGP) and live biotherapeutic product (LBP). The term 'psychobiotic' is commonly used in the literature to refer to a product providing health benefits regarding the nervous system and which has potential in treatment of neurological disorders (Cheng et al. 2019; Munawar et al. 2021; Sharma et al. 2021). However, the authors of this article believe that the proposed definition of psychobiotic does not present enough differences to justify it as a separate biotic (Zhu et al. 2023; Chiano et al. 2024). The distinction merely narrows the health benefit to the mental health, thus psychobiotics fall under the broader definition of probiotics (Hill et al. 2014). Since this issue has already been addressed in relation to probiotics, the discussion will now focus on NGP and LBP.

NGPs are described as new microbial strains isolated using culture independent methods, primarily genome sequencing. There is no unified and common definition; authors only often present differences between NGPs and conventional probiotics (Singh and Natraj, 2021; Abouelela and Helmy, 2024). Al-Fakhrany and Elekhnawy are one of few authors proposing the definition for NGP: "living microbes identified on the base of comparative microbiome investigations which confer health advantages to their host when taken to suitable extents" (Al-Fakhrany and Elekhnawy, 2024). This definition only narrows down the potential source of NGP, which is not restricted in any way by current definition of probiotic. The only difference that authors of this paper find compelling enough to consider the NGP as a separate group of biotics is the personalization of the preparations (Singh and Natraj 2021).

Live biotherapeutic product (LBP) is a term coined in the USA by Food and Drug Administration (FDA), to regulate the field of probiotics. It can be defined as: "a biological product that: 1) contains live organisms, such as bacteria; 2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and 3) is not a vaccine" (FDA, 2016). LBPs share more similarities with NGPs rather than with conventional probiotics. The context of application in treatment of a given disease, as stated in the second part of the definition, is crucial in the understanding the differences. Microorganisms do not have to exhibit specific health claim to be considered probiotics. According to the most recent probiotic definition, it is sufficient to demonstrate safety of use and general health benefits for the host, proven through human studies (Hill et al., 2014). Therefore, the terms LBP and probiotic cannot be used interchangeably, despite their similarities.

For stakeholders outside of the USA, the use of the term LBP may seem unjustified, given the presence of four biotics defined by ISAPP. Regardless, the term LBP is also used in EU, where its regulatory framework has been established in 2018 (Ph. Eur. 2018). Since probiotics are only required to demonstrate a general health benefit, the term LBP has been adopted to refer to products intended for the treatment or prevention of disease (Franciosa et al. 2023). This can be confusing since in Poland (member of EU) there are probiotics already functioning as drugs that aim to treat or prevent disease, which is not excluded by the ISAPP definition of probiotic (Hill et al., 2014; Ruszkowski et al. 2018).

3.1. Biotics complementary mode of action and health benefits

The interactions between probiotics, prebiotics, synbiotics, and postbiotics are complex and synergistic, lying in their complementary roles. As described before prebiotics enhance the growth of probiotics, synbiotics optimize the combined effects of probiotics and prebiotics, and postbiotics offer additional health benefits through their bioactive compounds. This interconnected relationship helps maintain a balanced gut microbiome, supports immune function, and improves overall health (see Figure 4).

The efficacy of biotics has been demonstrated in numerous randomised controlled trials (Andresen et al. 2020; Łukasik et al. 2022; Srivastava et al. 2024; Lau et al. 2024). Some biotics have been registered as drugs (see Table III and IV), further proving their effective-

ness. Although the positive effects of biotics are extensively studied, their direct mechanisms of action are often not fully understood. Human microbiota plays an important role in health and diseases, yet its complexity makes creating representative models to study the relations very challenging (El-Sayed et al. 2021; Rios Garza et al. 2023).

The bidirectional gut-brain axis plays an important role in maintaining homeostasis. The dysfunction of the axis has been shown in diseases such as irritable bowel syndrome (IBS), major depressive disorder or ASD (Socała et al. 2021; Hillestad et al. 2022). Administration of probiotics can positively influence the abnormal functioning of the axis through both direct and indirect interactions. Production of bioactive compounds such as serotonin or SCFA and interaction with enteric and autonomic nervous system, are possible ways in which probiotics can positively affect the axis (Mayer et al. 2022). The high abundance of microbiota in various body sites, particularly in the colon, is the principle behind the colonization resistance. In health, body sites are colonized by symbiotic microorganisms, inhibiting the colonization of pathogens – Figure 4 (Caballero-Flores et al. 2023). When this state is disturbed, body sites can be colonized by pathogens, leading to disease. Administration of probiotics can prevent the colonization of pathogens and help restore proper microbiota by colonizing the body sites themselves and/or promoting the colonization of other commensal microorganisms (Osbelt et al. 2021; Zheng et al. 2021; Gao et al. 2021). Prebiotics may also positively affect the integrity of the barrier by influencing the microbiota composition, significantly increasing the abundance of beneficial bacteria (Mellai et al. 2024).

Microorganisms present in GI tract are responsible for production and synthesis of various compounds, such as serotonin, gamma-aminobutyric acid (GABA), SCFA and vitamins (Beane et al. 2021; Socała et al. 2021; O'riordan et al. 2022). When the composition of microbiota is altered, an imbalance described as dysbiosis can occur. Administration of probiotics and their ability to produce SCFA, which lower the pH in the gut, can prevent the colonization of pathogens – Figure 4. SCFA are also used by the colonocytes as a source of energy (O'riordan et al. 2022). Postbiotics and synbiotics can also help in restoring the proper microbial composition e.g. by increasing the abundance of the *Faecalibacterium, Anaerobutyricum* and *Lactobacillales,* respectively (Jung et al. 2022; Srivastava et al. 2024; Naghibi et al. 2024). The microbiota plays crucial role in tryptophan and serotonin metabolism (Roth et al. 2021). Use of biotics can help maintain the proper balance, preventing dysbiosis, and when such imbalances occur, probiotics can help restore the balance (El-Sayed et al. 2021; He et al. 2022).

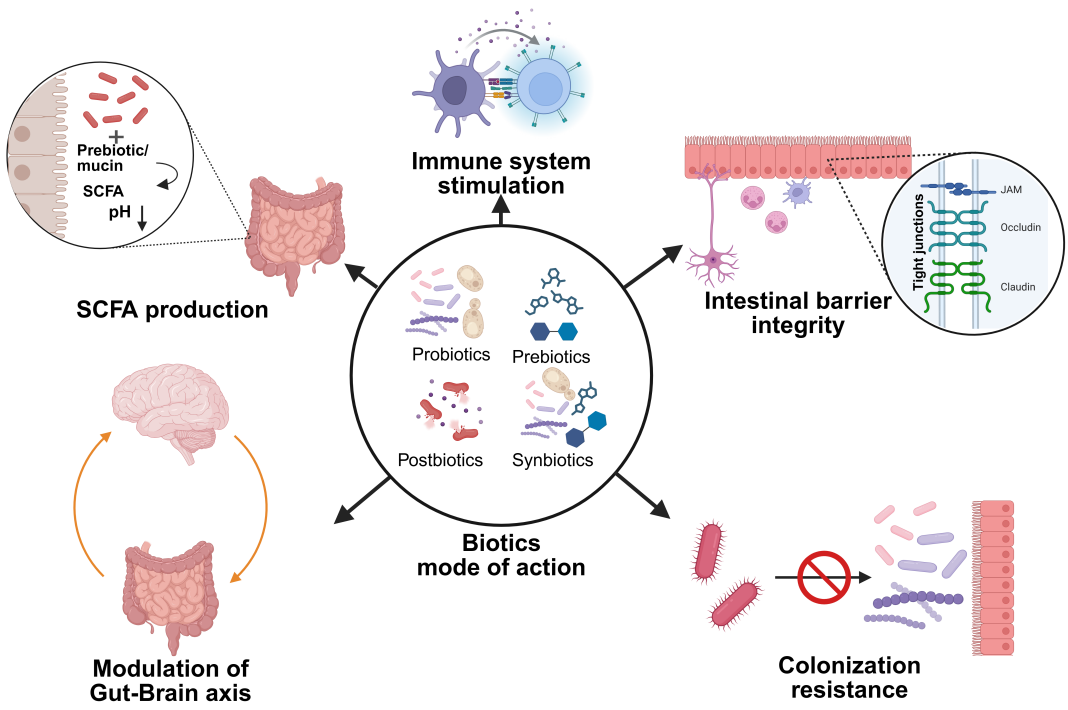

Figure 4. Basic mode of action of biotics. Created in BioRender.

The integrity of intestinal barrier is another very important aspect, which can be positively affected by biotics. In health, properly functioning barrier prevents the pathogens from penetrating the intestine wall and entering other body sites. Biotic administration, such as synbiotics, can enhance the integrity of the barrier by decreasing the level of pro-inflammatory biomarkers and increasing anti-inflammatory cytokines (Li et al. 2023). Mucin layer present in intestines prevents the direct contact of microorganisms with epithelial cells (Di Tommaso et al. 2021). Lack or thinning of this layer, observed in diseases e.g. inflammatory bowel disease (IBD), leads to constant stimulation of immune system as epithelial cells are directly exposed to microbial antigens. As a result, inflammation is observed and the integrity of the intestinal barrier is disrupted (Aleman et al. 2023). Mucin degradation is generally considered as a pathogenicity factor, but probiotic microorganism can use this ability to set an equilibrium between the mucin degradation and host production of mucin (Markowska and Kiersztan 2021). Products of mucin degradation, such as SCFA, can be beneficial to host. SCFA promote the tight junction formation, directly affecting the integrity of intestinal barrier (Hays et al. 2024). Constant immune system interactions with multiple microbial antigens, due to a disrupted intestinal barrier, negatively affect the host and can lead to diseases such as leaky gut syndrome (Chae et al. 2024). However, the interactions between the immune system and microorganisms are not always unfavourable. Postbiotic preparations can positively affect the activity of immune cells, thereby boosting host immunity (Kato et al. 2024).

While biotics offer a wide range of health benefits, the administration of probiotics and synbiotics can be associated with certain risks in immunocompromised individuals (Katkowska et al., 2021). In such populations, conditions like sepsis or endocarditis have been reported (Rahman et al., 2023; Eze et al., 2024). A promising alternative to mitigate these risks is the use of postbiotics (Figure 3). Preparations containing inanimate microorganisms, with or without their metabolites, do not carry the same risk associated with the intake of live microbes found in probiotics and synbiotics. Nevertheless, safety considerations remain essential, as components such as cell wall fragments or membrane elements e.g., endotoxin A (a part of the outer membrane in Gram-negative bacteria), may still raise significant safety concerns (Salminen et al., 2021; Vinderola et al., 2022). Changing the legal status of probiotics to medicinal products could further enhance their safety profile, as any contraindications, supported by clinical trials, would be required to be clearly disclosed.

3.2. Molecular pathways

As mentioned before, biotics can interact with host in various ways. In this section, we present two examples of probiotic-host interactions, focusing on *L. rhamnosus* GG and *A. muciniphila* MucT. The former strain represents conventional probiotics and the latter serves as an example of novel probiotic strain.

In a healthy gut, microorganisms rarely interact directly with the intestinal epithelium, with Payer's Patches being one of the few exceptions. This is primarily due to the protective mucin layer covering the epithelial surface. *L. rhamnosus* GG secrets proteins (most notably p40 and p75) that contribute to host health, with p40 exerting a more pronounced effect. p40 activates the epidermal growth factor receptor, leading to reduced apoptosis and enhanced mucus production – Figure 5A. These effects collectively strengthen intestinal barrier integrity, which is essential in maintaining homeostasis (Leser and Baker, 2024). Although indirect interactions via secreted proteins are critical, direct contact also plays a role. The expression of *Spa*CBA operon, encoding SpaCBA pili, by *L. rhamnosus* GG facilitates adhesion to host cells, thereby preventing pathogen adhesion through colonization resistance – Figure 5A (Spacova et al., 2020). Additionally, molecular interactions of *L. rhamnosus* GG with enterocytes can inhibit the formation of reactive oxygen species (ROS) and chloride ion excretion, counteracting two key pathogenic mechanisms of rotavirus infection (Buccigrossi et al., 2022). *A. muciniphila* MucT interacts with host via Amuc_1100 pili protein, which is recognized by Toll-like receptor 2 (TLR2) and lipooligosaccharide (LOS), which engages both TLR2 and Toll-like receptor 4 (TLR4) (Segers and de Vos, 2023; Garcia-Vello et al., 2024). These interactions enhance the transepithelial electrical resistance (TEER) and stimulate the production of anti-inflammatory cytokines such as IL-10, improving intestinal barrier integrity – Figure 5B (Ottman et al., 2017). *A. muciniphila* MucT indirect interactions are mediated by extracellular vesicles (EV) which also activate TLR2 and TLR4. The heat stable nature of LOS, EV and other components e.g. ornithine lipids, underscores its potential as a postbiotic (Garcia-Vello et

al., 2024; Ioannou et al., 2024). Another key aspect of this Gram-negative bacterium is mucin degradation. Through the activity of to various fucosidases and sialidases, *A. mucniphila* effectively degrades mucin, thus stimulating its turnover and promoting the growth of other beneficial microorganisms (Shuoker et al., 2023).

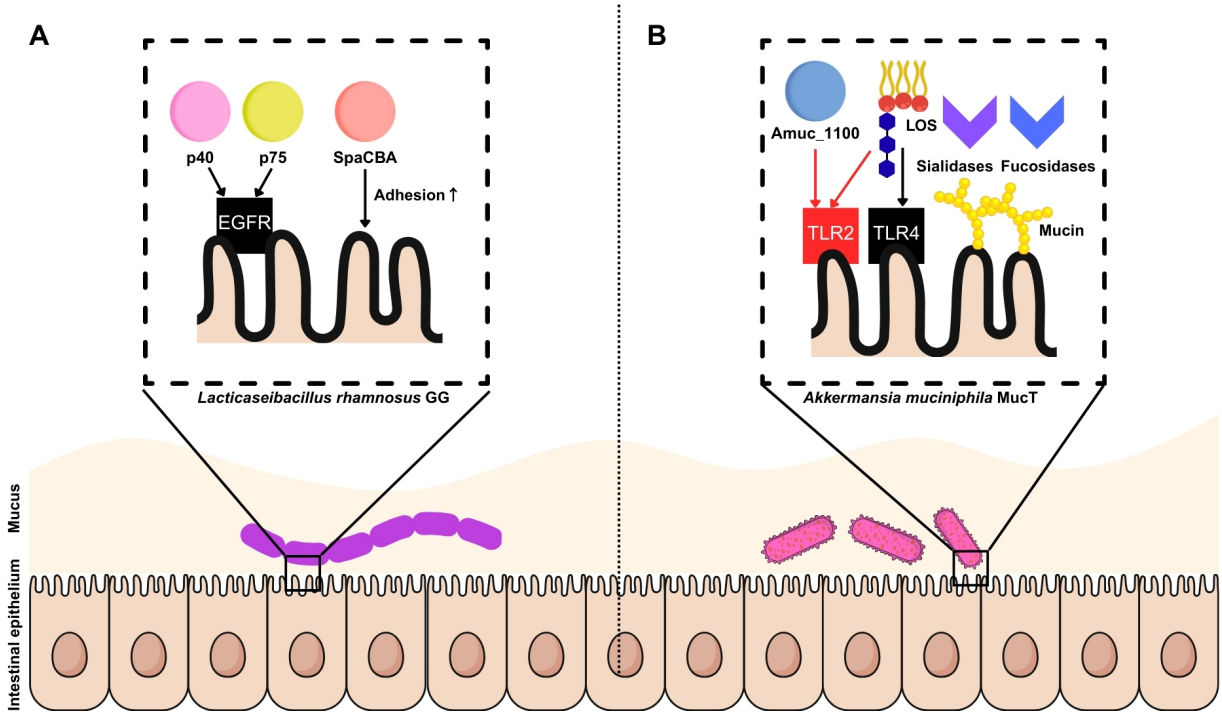

Figure 5. Selected molecular mechanisms by which two probiotic strains, A - *Lacticaseibacillus rhamnosus* GG and B –*Akkermansia muciniphila* MucT, interact with host intestinal epithelium. Panel A illustrates *L. rhamnosus* GG indirect interactions mediated by p40 and p75, which interact with EGFR, as well as direct interactions induced by SpaCBA. Panel B illustrates *A. muciniphila* MucT direct and indirect interactions, the former shown as Amuc_1100 and LOS interactions with TLR2 and TLR4, the latter as sialidases and fucosidases degrading mucin.

Legend: Amuc_1100 – *A. muciniphila* MucT pilus protein; LOS – lipooligosaccharide; TLR2 – Toll-like receptor 2; TLR4 – Toll-like receptor 4; p40/p75 – *L. rhamnosus* GG secreted proteins; EGFR - epidermal growth factor receptor; SpaCBA - *L. rhamnosus* GG pilus protein.

3.3. Single vs. multiple-strain probiotics

The difference in efficacy between multiple-strain probiotics and single-strain probiotics is not clear and seems to depend on the given strain(s) and their estimated outcomes rather than a general rule (Ouwehand et al. 2018). A meta-analysis conducted by McFarland shows that a two-strain probiotic containing *L. rhamnosus* GG and *B. lactis* Bb12, was more effective in eradicating the *H. pylori* than either strain alone. It was also found that single strain probiotic, containing *L. rhamnosus* GG was more effective in preventing necrotizing enterocolitis (NEC) compared with multiple strain probiotic containing the same strain. In cases of antibiotic-associated diarrhoea (AAD), atopic dermatitis/eczema, atopic dermatitis/allergy, upper respiratory tract infection (URTI), irritable bowel syndrome (IBS), there were no significant differences between single and multiple strain probiotics, whether the formulations were found to be effective or ineffective (McFarland, 2021). Another meta-analysis has shown superior effect of multiple strain probiotics in prevention of NEC (Morgan et al., 2020). Niu and Xiao's meta-analysis shows the superior effect of multiple strain probiotics in treatment of IBS, yet there are limitations to the study due to heterogeneity of RCTs (Niu and Xiao, 2020).

Evaluating the efficacy of single and multiple-strain probiotics is difficult, even when addressing the treatment or prevention of a specific disease. The number of papers that evaluate the differences between single and multiple-strain probiotics for the same strains is

limited. The differences in study design of RCTs (e.g. duration of treatment, dose), considering the same strain in different formulations, often prevent obtaining valuable data (McFarland 2021). Probiotics in multiple-strain formulations can exert additive, synergistic or antagonistic effects (Kwoji et al., 2021). Therefore, further research is needed to evaluate the efficacy of these formulations compared to corresponding single strain formulations, separately for a specific disease.

3.4. Efficacy and regulatory framework of biotics

Many clinical trials demonstrate the effectiveness of probiotics, prebiotics, synbiotics and postbiotics in various diseases (see Table II). Such use is particularly promising for diseases where current therapies prove to be ineffective or require long-term treatment. Given the critical role of the microbiota-gut-brain axis, biotics hold significant potential for managing psychiatric disorders, which are currently one of the major health challenges facing humanity (Socała et al. 2021).

The recent recognition that inanimate microorganisms can confer health benefits on the host, along with the unified definition of postbiotics presented by ISAPP, has facilitated studies and clinical trials for postbiotics (Salminen et al. 2021). Srivastava et al. studied the safety and efficacy of *Bifidobacterium longum* CECT 7347 as both a probiotic and postbiotic, the latter obtained through heat-treatment of the strain. The study proved safety and efficacy of both preparations, indicating that *Bifidobacterium longum* CECT 7347, in either form, is a good candidate for reducing the severity of IBS symptoms (Srivastava et al. 2024). The approach of studying the same strain in both probiotic and postbiotic formulation is uncommon and makes the study significant. The results show that postbiotics can be as effective as probiotics. In some aspects, postbiotics can be superior to probiotics, including better storage and safety standards (Ma et al. 2023; da Silva Vale et al. 2023).

The legal aspect of biotics is important considering their development and future. Regulations to classify a given biotic as a pharmaceutical or food supplement directly correspond to the quality of the product and its effectiveness. Currently the terms probiotic, prebiotic and synbiotic are overused (it is not the case for postbiotic since the term is novel). Many products, ranging from foods to personal care items, claim to contain probiotics. However, such statements are often not verified, due to the legal characteristics of these products. It is also important to acknowledge that the presence of live microorganisms in the product (e.g. in yogurt) is not enough to identify the product as a probiotic. Microorganisms present in such products must confer a proven health benefit to be considered probiotics (Hill et al. 2014).

The regulatory framework for probiotics is not homogenous across European Union. In Poland probiotics can be considered pharmaceuticals, food supplements and dietary foods for special medical purposes (Ruszkowski et al. 2018). In Poland there are only a few biotics registered as drugs – Table III and IV. Most of the biotics available commercially are food supplements, which do not undergo the strict regulations applied for drugs (Sionek and Kołożyn-Krajewska 2019).

In the USA, the FDA coined a new term, the live biotherapeutic product (LBP), to regulate the field of probiotics. The issue with this approach is that LBP can only refer to probiotics and synbiotics, as by the definition, LBP must contain live microorganisms (FDA 2016). To address postbiotics or prebiotics in a similar way, new term(s) must be coined, or LBP definition has to be modified.

The legal aspect of biotics is crucial in implementing safe and effective products that customers can trust. In addition to conducting the necessary studies to evaluate the safety and efficacy of probiotics, prebiotics, synbiotics, and postbiotics, it is important that the regulatory framework and laws adapt to the latest scientific literature, ensuring the access to high-quality products. The unification of the terms, such as those presented by ISAPP, should also be considered to enhance the customers knowledge (Liang et al. 2024).

Table II. Examples of health benefits demonstrated by probiotics, prebiotics, synbiotics and postbiotics in clinical trials.

Composition	Classification	Health benefits	Daily dose and duration	Reference
Lactiplantibacillus plantarum PS128	Probiotic	Amelioration of symptoms in children with ASD, such as: - disruptive and rule breaking behaviours - hyperactivity/impulsivity	$3x10^{10}$ CFU for 28 days	Liu et al., 2019
Bifidobacterium bifidum BGN4 *Bifidobacterium longum* BORI	Probiotic	- stress alleviation - mental flexibility - beneficial changes in microbiota	$1x10^{9}$ CFU $1x10^{9}$ CFU for 12 weeks	Kim et al., 2020
Lacticaseibacillus rhamnosus ŁOCK 0900 *Lacticaseibacillus rhamnosus* ŁOCK 0908 *Lacticaseibacillus casei* ŁOCK 0918	Probiotic	Significant improvement in atopic dermatitis symptom severity	$1x10^{9}$ CFU for 3 months	Cukrowska et al., 2021
Bifidobacterium bifidum W23 *Bifidobacterium lactis* W51 *Lactobacillus acidophilus* W37 *Lactobacillus acidophilus* W55 *Lacticaseibacillus paracasei* W20 *Lactiplantibacillus plantarum* W62 *Lacticaseibacillus rhamnosus* W71 *Ligilactobacillus salivarius* W24	Probiotic	Reduced risk of diarrhoea during and 7 days after antibiotic treatment	$1x10^{10}$ CFU during antibiotic treatment + 7 days	Łukasik et al., 2022
Bacillus subtilis BS50	Probiotic	Alleviation of gas-related gastrointestinal symptoms	$2x10^{9}$ CFU for 6 weeks	Garvey et al., 2022
Bacillus subtilis MB40	Probiotic	Elimination of *Staphylococcus aureus* without altering the microbiota	$1x10^{10}$ CFU for 30 days	Piewngam et al., 2023
Lacticaseibacillus rhamnosus CECT 30031, *Arthrospira platensis* BEA_IDA_0074B	Probiotic	Significant reduction in the severity of acne vulgaris	$1x10^{9}$ CFU for 12 weeks	Eguren et al., 2024
Escherichia coli Nissle 1917	Probiotic	Potential use of engineered *E. coli* Nissle 1917 in adenoma diagnosis and therapy of colorectal cancer	$1x10^{9}$ CFU for 14 days	Gurbatri et al., 2024
Saccharomyces boulardii CNCM I-745	Probiotic	In patients with SIBO, associated with dietary advice: - Improved digestive symptoms - restoration of the intestinal microbiota	500 mg for 15 days	Bustos Fernández, Man and Lasa, 2023
Streptococcus thermophilus BT01	Probiotic	Reduction of urease activity in faecal samples	$1x10^{11}$ aFU for 1 week	Martinović et al., 2023
Lactobacillus crispatus DSM32717 DSM32720, DSM32718, DSM32716	Probiotic	Reduction of the signs and symptoms of bacterial vaginosis - significant increase in the lactobacilli counts in the vagina - lowered combined score of the amount of discharge and itching/irritation in vulvovaginal candidiasis	$3x10^{10}$ CFU for 3 months	Mändar et al., 2023
Lactobacillus acidophilus *Lactiplantibacillus plantarum* *Bifidobacterium lactis* *Saccharomyces boulardii* (LactoLevure^{R})	Probiotic	- positive effects on glycaemic and lipid parameters - improvements in measures of adiposity in individuals with Type 2 Diabetes	$1,75x10^{9}$ CFU $0,5x10^{9}$ CFU $1,75x10^{9}$ CFU $1,5x10^{9}$ CFU for 6 months	Zikou et al., 2023
Lacticaseibacillus rhamnosus GG	Probiotic	Beneficial modulation of gut and skin microbiome	$1x10^{10}$ CFU for 12 weeks	Carucci et al., 2022

Composition	**Classification**	**Health benefits**	**Daily dose and duration**	**Reference**
Bifidobacterium longum CECT 7347	Probiotic	Reducing IBS symptom severity	$1x10^9$ for 84 days	Srivastava et al., 2024
Opuntia ficus-indica extract (Odilia™)	Prebiotic	Positive modulation of gut microbiota composition: - significant reduction in the *Firmicutes* to *Bacteroidetes* ratio - significant increase in relative abundances of beneficial bacteria - significant reduction in pro-inflammatory bacteria	300 mg for 8 weeks	Mellai et al., 2024
Inulin and oligofructose	Prebiotic	- significant improvement in frailty and renal function - increases in protein levels, body fat percentage, walking speed, grip strength - elevation in gut probiotic count - induced alterations in microbial metabolite expression levels among the older population	15 g for 3 months	Yang et al., 2024
Yeast mannan	Prebiotic	- An increase in the frequency and volume of bowel movements - accelerated transition to deep sleep stage and lengthened duration	1,1 g for 4 weeks	Tanihiro et al., 2024
Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium longum and galactooligosaccharides, xylooligosaccharides, resistant dextrin (SIM01)	Synbiotic	Alleviation of multiple symptoms of PACS	$2x10^{10}$ CFU for 6 months*	Lau et al., 2024
Bifidobacterium lactis HN019, Lacticaseibacillus rhamnosus HN001 and fructooligosaccharide	Synbiotic	Decrease in pro-inflammatory biomarkers (CRP and IFN-γ) and increased anti-inflammatory cytokine (IL-10 and sIgA)	$1,5x10^8$ CFU $7,5x10^7$ CFU and 500 mg for 8 weeks	Li et al., 2023
Lacticaseibacillus rhamnosus FloraActive™ 19070-2, *Lactobacillus acidophilus* DSMZ 32418, *Bifidobacterium lactis* DSMZ 32269, *Bifidobacterium longum* DSMZ 32946, *Bifidobacterium bifidum* DSMZ 32403 and fructooligosaccharides	Synbiotic	Significant amelioration in: - feeling of incomplete bowel movements - flatulence - pain - stool pressure and diarrheal stools	$1,96x10^9$ CFU $9,80x10^8$ CFU $5,88x10^9$ CFU $5,88x10^8$ CFU $5,88x10^8$ CFU and 1,894 g for 8 weeks	Skrzydło-Radomańska et al., 2020
Lactobacillus acidophilus La-14, Lactiplantibacillus plantarum Lp-115, Bifidobacterium animalis subsp. lactis CBG-C10 and fructooligosaccharide (LactominPlus®)	Synbiotic	- improvement in the degree of formed stool - decrease in faecal calprotectin level - increase in *Lactobacillales*	$2,9x10^7$ CFU $4,7x10^7$ CFU $2,4x10^7$ CFU and 1,2 g for 8 weeks	Jung et al., 2022
Bifidobacterium bifidum MIMBb75	Postbiotic	Alleviating IBS and its symptoms	$1x10^9$ cells for 8 weeks	Andresen, Gschossmann and Layer, 2020

Composition	Classification	Health benefits	Daily dose and duration	Reference
Limosilactobacillus reuteri DSM17648 (Pylopass)	Postbiotic	Improved effectiveness of *Helicobacter pylori* eradication therapy in patients with functional dyspepsia	$2x10^{10}$ cells for 28 days	Ivashkin et al., 2024
Bifidobacterium longum CECT 7347	Postbiotic	- decreased total and non-HDL cholesterol - significant increase in the abundance of the genera *Faecalibacterium* and *Anaerobutyricum* - reduced IBS symptom severity	$2,5x10^{9}$ cells for 8 or 12 weeks	Naghibi et al., 2024; Srivastava et al., 2024
Akkermansia muciniphila HB05	Postbiotic	Significant increase in muscle strength among individuals aged 60 years or older	$1x10^{10}$ cells for 12 weeks	Kang et al., 2024
Lacticaseibacillus paracasei MCC1849	Postbiotic	- increasing plasmacytoid dendritic cells activity - beneficial effects on immune cells in healthy adults	$5x10^{10}$ cells for 4 weeks	Kato et al., 2024

Legend: ASD – autism spectrum disorder, SIBO - small intestinal bacterial overgrowth, PACS - post-acute COVID-19 syndrome, CRP - C-reactive protein, IFN-γ - interferon gamma, IL-10 - interleukin-10, sIgA - secretory immunoglobulin A, IBS – irritable bowel syndrome, non-HDL - non–high-density lipoprotein, CFU – colony forming unit, aFU - active fluorescent unit, *- no data for prebiotic dose.

Table III. Orally administered probiotics and postbiotics, commercially available in Poland and registered as drugs.

Name	Classification	Content per one capsule or sachet	Recommended use
Lakcid Forte - POLPHARMA S.A.	Probiotic	$10x10^{9}$ CFU: - *Lacticaseibacillus rhamnosus* Pen (40%) - *Lacticaseibacillus rhamnosus* E/N (40%) - *Lacticaseibacillus rhamnosus* Oxy (20%)	Treatment of antibiotic-associated colitis, including pseudomembranous colitis; supportive treatment during and after antibiotic therapy; prevention of traveller's diarrhoea
Lakcid Entero - POLPHARMA S.A.	Probiotic	250 mg ($\geq 10^{10}$ CFU/1 g) - *Saccharomyces cerevisiae var. boulardii*	Treatment of acute infectious diarrhoea, diarrhoea in IBS, AAD, recurrent *Clostridium difficile* diarrhoea; prevention of diarrhoea associated with enteral nutrition, traveller's diarrhoea, as an adjunct in treatment of *H. pylori*
Lacidofil - LALLEMAND S.A.S.	Probiotic	$2x10^{9}$ CFU*: - *Lacticaseibacillus rhamnosus* R0011 - *Lactobacillus helveticus* R0052	Treatment of recurrent pseudomembranous colitis, supportive treatment during and after antibiotic therapy; prevention of traveller's diarrhoea
Enetrol – BIOCODEX	Probiotic	250 mg: - *Saccharomyces boulardii* CNCM I-745	Treatment of acute infectious diarrhoea, recurrent *Clostridium difficile* diarrhoea; prevention of diarrhoea associated with enteral nutrition, traveller's diarrhoea; as an adjunct in treatment in IBS diarrhoea
Lacteol Fort 340 mg - DSM-Firmenich Houdan SAS	Postbiotic	340 mg including: - Inactivated *Limosilactobacillus fermentum* and *Lactobacillus delbrueckii* – $10x10^{9}$ CFU - Fermented medium – 160 mg	Supportive treatment of diarrhoea
Trilac - Krotex Pharm	Probiotic	$1,6x10^{9}$ CFU: - *Lactobacillus acidophilus* La-5 (37,5%) - *Lactobacillus delbrueckii* subsp. *bulgaricus* Lb-Y27 (25%) - *Bifidobacterium animalis* subsp. *lactis* Bb-12 (37,5%)	Treatment of antibiotic-associated colitis, including pseudomembranous colitis; prevention of traveller's diarrhoea; supportive treatment after antibiotic therapy

Legend: AAD – antibiotic-associated diarrhoea; CFU – colony forming unit; IBS – irritable bowel syndrome; * - ratio for each strain has not been declared.

Table IV. Non-orally administered probiotics registered as drugs in Poland.

Name	Classification	Content per one capsule	Recommended use
Lakcid Intima – POLPHARMA S.A.	Probiotic	- *Lactobacillus gasseri* DSM 14869 ≥10^8 CFU - *Lacticaseibacillus rhamnosus* DSM 14870 ≥10^8 CFU	Preventive use to maintain or restore normal vaginal microbiota
Lactovaginal – BIOMED S.A.	Probiotic	- *Lacticaseibacillus rhamnosus* 573 ≥10^8 CFU	Preventive use; treatment of vaginal discharge and inflammation of reproductive organs after the antibacterial, antitrichomonal, or antifungal treatment
inVag – BIOMED S.A.	Probiotic	≥10^9 CFU: - *Limosilactobacillus fermentum* 57A (25%) - *Lactiplantibacillus plantarum* 57B (25%) - *Lactobacillus gasseri* 57C (50%)	Prevention of genitourinary infections; supportive treatment in vaginitis, during and after antibiotic and/or antifungal treatment
Protrivagin – Verco S.A.	Probiotic	- *Lactiplantibacillus plantarum* P 17630 10^8 CFU	Normalization of the disrupted vaginal microbiota after antibiotic therapy for bacterial vaginosis; maintaining normal vaginal microbiota in recurrent infections

Legend: CFU – colony forming unit.

4. Future perspectives

Biotics present great potential in treatment and prevention of multiple diseases. As mentioned in the previous paragraph, the regulatory framework can be a limiting factor for implementing novel therapeutics. Therefore, the future of biotics greatly depends on legal aspects (Cordaillat-Simmons et al. 2020; Liang et al. 2024).

Some authors point out that individual differences in microbiota make the use of formulations with invariable composition unjustified (Lee et al. 2021). This has led to the idea of using personalized therapies. Such personalization could be achieved based on the presence of the characteristic microbiota. In 2011 the idea of enterotypes was proposed (Arumugam et al. 2011). The study distinguished three enterotypes based on specific relation of the present taxa. Since then, the idea of enterotypes has been studied. Multiple authors proposed a new insight on the topic, considering new classification, the influence of enterotypes on nutrition and probiotic intake (Costea et al. 2017; Liang et al. 2017; Chen et al. 2017; Song et al. 2020; Lee et al. 2021; Cerdó et al. 2022; Yuan et al. 2022) Although the idea of enterotypes is well established in the literature, novel reports show no basis for identifying such groups, thus suggesting the absence of enterotypes in the human gut (Bulygin et al. 2023).

While the idea of enterotypes evolved and number of distinguished enterotypes has changed, the approach to question their existence in general, as presented by Bulygin et al., is novel and groundbreaking (Gorvitovskaia et al. 2016; Mobeen et al. 2018; Jiao et al. 2022; Bulygin et al. 2023). To the best of authors knowledge, the cited article is the only one that states the absence of enterotypes and supports this claim with data (Bulygin et al. 2023). The idea of enterotypes, understood as discrete clusters, was challenged earlier by Cheng and Ning, who proposed a more continuous understanding of the term (Cheng and Ning 2019).

Such cutting-edge approach, denying the existence of enterotypes, may be controversial given the fact that the idea of enterotype has been well established in the literature. Many clinical trials proved the corelation between the enterotypes and health (Christensen et al. 2020; Vallet et al. 2023; Jamieson et al. 2024).

As our understating of human microbiota constantly evolves, the idea of personalized therapies can be promising, even if enterotypes will be abandoned in their present understanding (Abouelela and Helmy 2024). Tools such as next-generation sequencing and machine learning help isolate potentially beneficial

microorganisms, by some classified as NGP, and at the same time provide more data for better understanding of the microbiota relations (Chollet et al. 2024; Hasnain et al. 2024). The field of biotics would greatly benefit from the unification of nomenclature, a problem this article directly addresses. The wide variety of terms used, often synonymous, hinders the understanding of the subject by stakeholders (Salminen et al. 2021). Biotics are promising in the treatment of various diseases, including civilization diseases, positively affecting general health, preventing colonization of the pathogens and dysbiosis (see Table II) (Logan and Katzman 2005; Maldonado Galdeano et al. 2019; Osbelt et al. 2021; Caballero-Flores et al. 2023).

Further research could focus on postbiotic inactivation methods. As shown, inanimate microorganisms and their metabolites exhibit great potential, which is often limited by the lack of efficient inactivation techniques, capable of preserving bioactive properties, while remaining cost-effective and scalable. Additionally, omics-driven approaches may be employed to identify novel probiotic candidates and to investigate the characteristics and potential applications of already selected strains. Characterization of individual microbiome using next-generation sequencing (NGS) can enable the development of personalized therapies. As shown, microorganism derived products such as secreted proteins can exert therapeutic effect. Studying the proteomics on both host and microbial level along with their interactions, may deepen our understanding of host-microbiome relationship, supporting the development of novel biotics. Evaluating the efficacy of biotics, such as differences between single- and multi-strain probiotics, safety considerations, and the regulatory framework, remains a critical area of research.

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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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REVIEW
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OCTENIDINE DIHYDROCHLORIDE – ANTIMICROBIAL ACTIVITY, ADAPTATION AND CLINICAL APPLICATION

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Abstract: Octenidine dihydrochloride (OCT) is an antiseptic used for the prevention of wound infections, treatment of wounds and for treating oral infections. The spectrum of OCT's activity includes Gram-positive and Gram-negative bacteria, as well as fungi, including multidrug-resistant (MDR) strains. For most species, it exhibits activity at concentrations ranging from approximately 1 to several µg/mL. OCT also exhibits strong antibiofilm activity, both against biofilm formation and mature biofilms. The compound has limited virucidal and antiparasitic activity. The Clinical Efficiency of MIC (CEMIC) index for most pathogens is classified as excellent, meaning that the MIC is much lower than the clinical concentration. The required contact time for OCT microbicidal action is fast, at just 1 minute. The possibility of adaptation to OCT has been described; however, the Karpinski Adaptation Index (KAI) for most species is below 0.2, indicating a very low or low risk of developing clinical resistance. Only in some isolates of *Proteus mirabilis* and *Pseudomonas aeruginosa* the risk of resistance development considered moderate. According to guidelines (Statement of the Polish Wound Management Association, German Consensus on Wound Antisepsis, and International Consensus Document "Use of wound antiseptics in practice"), OCT is the first-choice antiseptic for critically colonized wounds, infection-prone wounds, burns, wounds colonized by multidrug-resistant (MDR) pathogens or infected wounds, and for the prevention of surgical site infections (SSI). OCT is also used in umbilical stump care, the treatment of oral infections, skin and mucosal candidiasis, and bacterial vaginosis.

1. Introduction. 2. Mode of action, 3. Antimicrobial activity, 4. Antibiofilm activity, 5. Bactericidal time, 6. Adaptation to OCT, 7. Precautions and application of OCT.

Keywords: anti-infective agents; antimicrobial stewardship; biofilm; nosocomial infection; wound infection; wound healing;

1. Introduction

A growing concern is the increasing number of individuals with wounds. It is estimated that approximately 1–2% of people worldwide experience chronic wounds (Sharma et al. 2024). An additional threat is the rise of multidrug-resistant (MDR) bacterial and fungal strains, leading to therapeutic failure and becoming a serious crisis (Bharadwaj et al. 2022; Bonomo et al. 2024). According to the latest guidelines (Nair et al. 2023; Sopata et al. 2023) antibiotics should be preserved, and antiseptics should be used for wound prevention and treatment. Antiseptics are antimicrobial agents that act at various levels: on the wound surface, in exudate, within the dressing structure, and in tissues. One such antiseptic is octenidine.

Octenidine dihydrochloride (OCT) is a cationic compound, stable within a pH range of 1.6–12.2 (Hübner et al. 2010). It has PubChem CID 51167, its molecular weight is 623.8 g/mol, and molecular formula $C_{36}H_{64}Cl_2N_4$ (PubChem). It exhibits strong antimicrobial activity, including effectiveness against Gram-pos-

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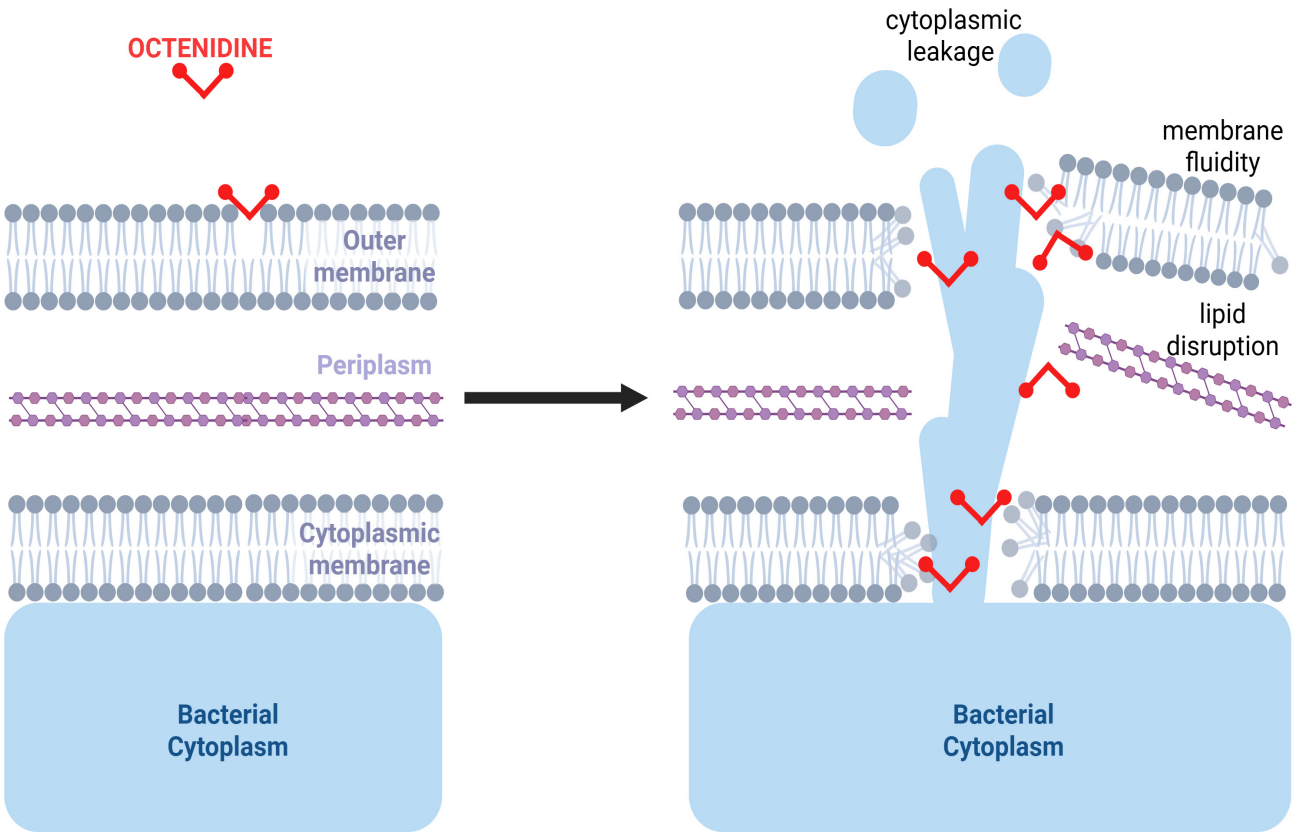
itive and Gram-negative bacteria, fungi, some viruses, and protozoa, while maintaining low cytotoxicity. OCT was introduced into medical practice over 25 years ago and is currently used in washing lotions, mouth rinses, oral tablets, and skin disinfectants.

2. Mode of action

OCT interacts with bacterial polysaccharides and enzymatic systems, leading to cytoplasmic leakage and disruption of essential cellular functions (Hübner et al. 2010). Unlike antibiotics that target specific cellular components, OCT exerts its antimicrobial effect by destabilizing the cell structure, compromising membrane integrity, disrupting the lipid bilayer, and increasing membrane permeability (Vejzovic et al. 2022). Additionally, it neutralizes the bacterial surface charge, causing the outer membrane to rupture and the cell wall to degrade. Once inside the periplasmic space, OCT reaches the inner membrane, where it induces lipid disruption, leading to depolarization and changes in membrane fluidity (Figure 1) (Malanovic et al. 2020). In *Candida* species, OCT has been shown to inhibit filamentation by interfering with ergosterol biosynthesis and compromising membrane integrity (Fang et al. 2023). Since its mechanism of action does not rely on lipid specificity, it is effective against a broad range of bacteria and fungi, including MDR strains (Malanovic et al. 2022). Due to its nonspecific mode of action, which involves membrane disruption, the likelihood of resistance development is considered minimal, and no cases of OCT resistance have been reported in clinical practice (Malanovic et al. 2020).

Figure 1. Mode of action of octenidine dihydrochloride. Created using the BioRender.com.



3. Antimicrobial activity

OCT exhibits a strong antibacterial effect (Koburger et al. 2010; Dydak et al. 2021; Krasowski et al. 2021; Loose et al. 2021; Denkel et al. 2022; da Silva et al. 2023). However, it has no effect on bacterial spores (Bigliardi et al. 2017). The minimal inhibitory concentrations (MIC) for most tested bacteria range from below 1 μg/mL to approximately 10 μg/mL (Table 1). However, for single strains of *Streptococcus pneumoni-*

ae and *Pseudomonas aeruginosa*, the maximum MIC values are significantly higher, at 32 μg/mL and 80 μg/mL, respectively. Fungi show similar susceptibility to OCT, with MIC values ranging from approximately 0.5 to 4 μg/mL. These MIC levels indicate that the antiseptic is effective at similar concentrations across different species. Comparable inhibitory concentrations of OCT have also been observed in MDR strains such as New Delhi metallo-β-lactamase-positive (NDM) *Enterobacter cloacae*, *Klebsiella pneumoniae* NDM, and *Candida auris* (Karpiński et al. 2025a). Additionally, for all MIC results, the Clinical Efficiency of MIC (CEMIC) index was analyzed, which represents the ratio of MIC values to clinical concentrations (Karpiński, et al. 2025b). The lowest clinical concentration of OCT used is 500 μg/mL. CEMIC is classified as excellent for values < 0.1, moderate for values between 0.1 and 0.9, and poor for values > 0.9 (Karpiński, et al. 2025b). For most species listed in Table 1, CEMIC was classified as excellent, meaning the MIC is much lower than the clinical concentration. This is particularly important for antiseptics, which, for example, may become diluted in wounds due to exudate or blood. In the case of OCT, even significant dilution within the wound does not reduce its activity. However, for some *P. aeruginosa* strains, CEMIC was classified as moderate.

Table 1. Minimal inhibitory concentrations (MIC) of octenidine against bacteria and fungi using microdilution method.

Microorganisms	Range of MICs (μg/mL)	Methodological remarks (medium type, colony counts, incubation time, and temperature)	References
Gram-positive bacteria			
Clostridium perfringens	1	MHB, 10^5 cfu/mL, 24-48 h, 36°C	(Koburger et al. 2010)
Enterococcus faecalis	4	MHB, 10^5 cfu/mL, 24-48 h, 36°C	(Koburger et al. 2010)
	3.125-6.25	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
E. faecium	0.49-1.95	TSB, 10^5 cfu/mL, 24 h, 37°C	(Dydak et al. 2021)
	3.125-6.25	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
E. hirae	0.6-10	TSB, 10^8-10^9 cfu/mL, 24-72 h, no data	(Schug et al. 2022)
Staphylococcus aureus	2	MHB, 10^5 cfu/mL, 24-48 h, 36°C	(Koburger et al. 2010)
	0.49-0.98	TSB, 10^5 cfu/mL, 24 h, 37°C	(Dydak et al. 2021)
	2-4	SCS, 1.5-5×10^5 cfu/mL, 48 h, 37°C	(Denkel et al. 2022)
	0.9	MHB, 10^5 cfu/mL, 24 h, 37°C	(Krasowski et al. 2021)
	0.3-5	TSB, 10^8-10^9 cfu/mL, 24-72 h, no data	(Schug et al. 2022)
	3.125-6.25	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Methicillin-resistant *S. aureus* (MRSA)	1	MHB, 10^5 cfu/mL, 24-48 h, 36°C	(Koburger et al. 2010)
	1-4	MHB, 5×10^5 cfu/mL, 24-48 h, 37°C	(Dittmann et al. 2019)
S. epidermidis	0.49-7.8	TSB, 10^5 cfu/mL, 24 h, 37°C	(Dydak et al. 2021)
Coagulase-negative staphylococci	2-4	SCS, 1.5-5×10^5 cfu/mL, 48 h, 37°C	(Denkel et al. 2022)
Streptococcus pneumoniae	8-32	MHB, 10^5 cfu/mL, 24-48 h, 36°C	(Koburger et al. 2010)
S. pyogenes	3.125-6.25	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Gram-negative bacteria			
Acinetobacter baumannii	0.25-3.9	TSB, 10^5 cfu/mL, 24 h, 37°C	(Dydak et al. 2021)
Enterobacter cloacae	3.9	TSB, 10^5 cfu/mL, 24 h, 37°C	(Dydak et al. 2021)
	6.25	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)

Escherichia coli	2	MHB, 10^5 cfu/mL, 24-48 h, 36°C	(Koburger et al. 2010)
	1.95-3.9	TSB, 10^5 cfu/mL, 24 h, 37°C	(Dydak et al. 2021)
	2-4	SCS, 1.5-5×10^5 cfu/mL, 48 h, 37°C	(Denkel et al. 2022)
	1.95-3.9	MHB or artificial urine, 10^5-10^6 cfu/mL, 20 ± 2 h, 37°C	(Loose et al. 2021)
	1-4	MHB, 10^6 cfu/mL, 20 ± 2 h, 37°C	(da Silva et al. 2023)
	0.6-20	TSB, 10^8-10^9 cfu/mL, 24-72 h, no data	(Schug et al. 2022)
	3.125-6.25	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Haemophilus influenzae	1	MHB 10^5 cfu/mL, 24-48 h, 36°C	(Koburger et al. 2010)
Klebsiella spp.	2-4	SCS, 1.5-5×10^5 cfu/mL, 48 h, 37°C	(Denkel et al. 2022)
K. pneumoniae	1.95-7.8	TSB, 10^5 cfu/mL, 24 h, 37°C	(Dydak et al. 2021)
	3.125-6.25	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Proteus mirabilis	1.95-3.9	MHB or artificial urine, 10^5-10^6 cfu/mL, 20 ± 2 h, 37°C	(Loose et al. 2021)
	3.125-6.25	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Pseudomonas aeruginosa	2-8	MHB, 10^5 cfu/mL, 24-48 h, 36°C	(Koburger et al. 2010)
	3.9-15.7	TSB, 10^5 cfu/mL, 24 h, 37°C	(Dydak et al. 2021)
	8-32	SCS, 1.5-5×10^5 cfu/mL, 48 h, 37°C	(Denkel et al. 2022)
	2.25±0.95	MHB, 10^5 cfu/mL, 24 h, 37°C	(Krasowski et al. 2021)
	3.9-7.8	MHB or artificial urine, 10^5-10^6 cfu/mL, 20 ± 2 h, 37°C	(Loose et al. 2021)
	3.91-15.63	TSB, 10^5 cfu/mL, 24 h, 36°C	(Karpiński, et al. 2025b)
	1.25-80	TSB, 10^8-10^9 cfu/mL, 24-72 h, no data	(Schug et al. 2022)
	3.125-12.5	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Salmonella enterica	6.25	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Shigella flexneri	6.25-12.5	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Yersinia enterocolitica	6.25	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Fungi			
Ascophera apis	0.78-3.125	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Candida albicans	1	MHB, 10^5 cfu/mL, 24-48 h, 36°C	(Koburger et al. 2010)
	0.49-0.98	TSB, 10^5 cfu/mL, 24 h, 37°C	(Dydak et al. 2021)
	0.45	RPMI with 2% glucose, 10^5 cfu/mL, 24 h, 37°C	(Krasowski et al. 2021)
	0.5 ± 0.25 and 0.9 ± 0.4	TSB, 10^6 cfu/mL, 24 h, 36°C	(Korbecka-Paczkowska and Karpiński 2024)
	1.95-3.91	Sabouraud broth, 10^6 cfu/mL, 24 h, 36°C	(Karpiński et al. 2024)
	0.78-1.56	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
C. auris	3.125	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
C. glabrata	0.78-3.125	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
C. tropicalis	0.78-1.56	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Cryptococcus neoformans	3.125	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)
Rhodotorula mucilaginosa	3.125	TSB, 10^5 cfu/mL, 24-48 h, 37°C	(Karpiński, et al. 2025a)

Abbreviations: MHB - Mueller–Hinton broth, TSB - Tryptic soy broth, SCS - Soybean casein solution, RPMI – Roswell Park Memorial Institute medium

OCT has limited virucidal activity, and the number of studies on this topic is scarce (Bigliardi et al. 2017). One of the studies reported that 0.1% concentration may be effective against coliphages f2 and MS2, as well as hepatitis B and herpes simplex viruses, but not against phages PhiX174 and adenoviruses (Hübner et al. 2010). The authors suggest that OCT exhibits virucidal activity only against enveloped viruses. However, there is a lack of recent studies confirming this effect.

The COVID-19 pandemic prompted several studies on the effect of OCT against SARS-CoV-2. In one study, using EN 14476 guidelines, a significant viral titre reduction was observed after 15 seconds of contact (Steinhauer et al. 2021). Another study showed that rinsing the mouth with OCT for one minute reduced SARS-CoV-2 RNA in saliva to undetectable levels by RT-qPCR (Smeets et al. 2022). However, both studies used Octenisept, which contains 0.1% OCT and 2% phenoxyethanol (PE). Since PE also has antimicrobial properties, it is difficult to attribute the antiviral effect solely to OCT. This is supported by a study where a product with 0.05% OCT but no PE showed weak activity against SARS-CoV-2 (Meister et al. 2020).

The antiparasitic effect of OCT has been described in relation to *Trichomonas vaginalis*. A combination of 0.1% OCT and 2% PE, demonstrated 50% effective concentration (EC50) values after 5 minutes of exposure at concentrations ranging from 5.7 to 21.4 µg/mL, and after 30 minutes at concentrations of 0.68 to 2.1 µg/mL (Küng et al. 2016). However, as with viruses, it remains unclear whether the anti-*Trichomonas* activity is primarily due to OCT, PE, or a combination of both.

4. Antibiofilm activity

OCT exhibits strong antibiofilm activity, both against biofilm formation and mature biofilm. Most studies show that complete biofilm reduction occurs within 24 hours, regardless of the microbial species (Rembe et al. 2020; Dydak et al. 2021; Krasowski et al. 2021; Loose et al. 2021). Only one publication showed that OCT requires up to 3 days for biofilm formation inhibition of *E. coli* (Loose et al. 2021). Table 2 demonstrates that the OCT concentrations required for antibiofilm activity are lower for Gram-positive bacteria than for Gram-negative bacteria. In the case of *C. albicans*, the data are inconclusive. There are publications describing the effect of OCT on bacterial viability in biofilms and biofilm reduction. Unfortunately, these data are very diverse. In some studies, OCT destroys 100% of the biofilm already at concentrations <100 µg/mL (Dydak et al. 2021; Krasowski et al. 2021), while in others, even a concentration of 1000 µg/mL does not destroy the entire biofilm (Davis et al. 2017; Rembe et al. 2020; Korbecka-Paczkowska and Karpiński 2024). It was also confirmed that OCT leads to the destruction of MRSA biofilm structure *in vivo* in mice (Huang et al. 2021). However, there is a lack of studies investigating its effect on the biofilm matrix in a short time. This would be important due to the short, usually only a few minutes long, application of OCT-containing products, such as oral mouthwashes or wound liquids.

Table 2. Antibiofilm activity of octenidine dihydrochloride.

Microorganism	Tested concentrations (µg/mL)	Time of action	% of biofilm reduction	Type of antibiofilm study	Reference
E. faecium	15.7-31.3	24 h	100	mature biofilm reduction	(Dydak et al. 2021)
S. epidermidis	15.7-125	24 h	100		(Dydak et al. 2021)
S. aureus	62.5	24 h	100		(Dydak et al. 2021)
	~50	24 h	100		(Krasowski et al. 2021)
	1000	24 h	~85%		(Rembe et al. 2020)
MRSA	1000	3 days	80%		(Davis et al. 2017)
A. baumannii	7.8-250	24 h	100		(Dydak et al. 2021)
E. coli	250	3 days	100	biofilm formation inhibition	(Loose et al. 2021)
	125-500	24 h	100	mature biofilm reduction	(Dydak et al. 2021)
E. cloacae	250-500	24 h	100		(Dydak et al. 2021)
K. pneumoniae	62.5-500	24 h	100		(Dydak et al. 2021)

P. mirabilis	250	24 h	100	biofilm formation inhibition	(Loose et al. 2021)
P. aeruginosa	500	24 h	100		(Loose et al. 2021)
	250 to >500	24 h	100	mature biofilm reduction	(Dydak et al. 2021)
	~180	24 h	100		(Krasowski et al. 2021)
	1000	24 h	~100		(Rembe et al. 2020)
C. albicans	500	24 h	47 ± 11		(Korbecka-Paczkowska and Karpiński 2024)
	1000	24 h	51 ± 13		(Korbecka-Paczkowska and Karpiński 2024)
	15.7-31.3	24 h	100		(Dydak et al. 2021)
	~60	24 h	100		(Krasowski et al. 2021)

5. Bactericidal time

According to the European Standard EN 1040:2005, an effective antiseptic should achieve a 5-log as below reduction of a given bacteria (European Standard EN 1040:2005). This corresponds to a 99.999% reduction in pathogen count. Studies indicate that pure OCT at a concentration of 500 μg/mL reduced the planktonic form of *P. aeruginosa* by over 5-log within 1 minute (Karpiński, et al. 2025b). In other studies, a significant reduction of *C. albicans*, *S. aureus*, and *P. aeruginosa* also required a contact time of 1 minute and OCT concentrations ranging from 10 to 50 μg/mL (Koburger et al. 2010). This contact time is shorter for the OCT/PE combination, e.g. for Octenisept with 1000 μg/mL of OCT. The contact time required for total inhibition of *S. aureus*, *E. faecalis*, and *C. albicans* is only 15 seconds, for this product. For a 50% solution, the contact time for *E. faecalis* and *C. albicans* increased to 3 minutes (Tirali et al. 2009). OCT/PE achieves a 5 $\log_{10}$ CFU/mL reduction within 1 minute against *P. aeruginosa* and *S. aureus* under standard conditions (EN 13727), in the presence of wound exudate, as well as in a modified peptide challenge test (Severing et al. 2022). Studies conducted in accordance with EN 13727:2012+A1 demonstrated that OCT at a concentration of 100 μg/mL achieves a reduction of >5 $\log_{10}$ within 1 minute for isolates of *A. baumannii*, *E. cloacae*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. This activity was observed in three types of media: without organic load, with albumin, and with albumin and erythrocytes (Alvarez-Marin et al. 2017). Some papers indicate that OCT may be less effective in the presence of organic material (Schedler et al. 2017; Barreto et al. 2020). Schedler et al. (2017) showed that, for 1000 μg/mL OCT, the time required for reduction of microorganisms by ≥ 5 $\log_{10}$ in the presence of organic soil can lasts from 3 h to 24 h.

Contact time in biofilm conditions needs to be extended. After 30 minutes of 500 μg/mL OCT exposure, 66.6% of *C. albicans* cells within the biofilm remain viable, while complete eradication occurs only after 1 hour. For *S. aureus* and *P. aeruginosa*, 66.6% of bacteria remain viable after 15 minutes, 55.5% after 30 minutes, and complete killing is achieved after 24 hours (Krasowski et al. 2021). In other studies, the OCT/PE combination eradicated bacterial viability in mature *P. aeruginosa* biofilm by 46% within 15 minutes and 100% within 30 minutes, while *S. aureus* was completely eradicated within 1 minute (Junka et al. 2014). The faster action may be associated with the additional presence of PE.

6. Adaptation to OCT

Adaptation to antiseptics is a process in which bacteria and/or fungi gradually increase their tolerance to a given antiseptic after repeated or prolonged exposure (Verspecht et al. 2019). This adaptation often leads to the ability of microorganisms to grow at increasing concentrations of antiseptics. In contrast to classical antibiotic resistance, adaptation to antiseptics usually does not result from the acquisition of resistance genes but rather from mechanisms such as biofilm formation, metabolic changes and growth retardation, alterations in cell membrane structure, and active removal of the antiseptic from the cell *via* efflux pumps (Verspecht et al. 2019; Wand et al. 2019; Bock et al. 2021). Wand et al. (Wand et al. 2019) described the efflux pump SmvA and membrane remodeling as responsible for OCT tolerance in *K. pneumoniae*. Additionally, it was observed that adaptation to chlorhexidine may lead to decreased susceptibility to other cationic bio-

cides, including OCT. Tolerance associated with the efflux pump has been linked to mutations in phosphatidylserine synthase *pssA* and phosphatidylglycerolphosphate synthase *pgsA* (Bock et al. 2021). In another study, opposite conclusions were drawn, demonstrating that Gram-positive bacteria carrying genes encoding efflux pumps contribute to antimicrobial resistance but do not affect sensitivity to low concentrations of OCT (Conceição et al. 2019). The results of studies on adaptation to OCT are varied. Table 3 shows that some studies found no development or only low tolerance to OCT in strains such as *S. aureus, S. epidermidis, Citrobacter* spp., and *Enterobacter* spp. (Nicolae Dopcea et al. 2020; Garratt et al. 2021; Karpiński 2024). However, other publications confirmed the development of adaptation to OCT, particularly in *P. mirabilis* and *P. aeruginosa* (Shepherd et al. 2018; Garratt et al. 2021; Pelling et al. 2024). The Karpinski Adaptation Index (KAI) is used in studies to assess the potential risk of developing resistance to antiseptics (Karpiński 2024). For most strains listed in Table 3, the KAI is below 0.2, indicating that the level of adaptation is significantly lower than the clinical concentration. Therefore, these strains have a very low or low risk of developing clinical resistance to OCT. Only in some isolates of *P. mirabilis* and *P. aeruginosa* does the risk of resistance development increase to a moderate level (Table 3).

Table 3. Results of studies on the development of microorganism adaptation to OCT.

Microorganism	Initial MIC (before adaptation) (µg/mL)	MIC after adaptation (µg/mL)	Fold increase in adaptation relative to initial MIC	Reference	Karpinski Adaptation Index (KAI)	Risk of clinical resistance to OCT
S. aureus	2	4.5	× 2.25	(Karpiński 2024)	0.009	Very low
S. epidermidis	0.2	0.49	× 2.45	(Nicolae Dopcea et al. 2020)	0.00098	Very low
Citrobacter spp.	2	2	× 1	(Garratt et al. 2021)	0.004	Very low
Enterobacter spp.	4	4-8	× 1-2	(Garratt et al. 2021)	0.008-0.016	Very low
P. mirabilis	2	128	× 64	(Pelling et al. 2024)	**0.256**	Moderate
	8	16	× 2	(Tagliaferri et al. 2024)	0.032	Very low
P. aeruginosa	7.8–15.6	50-75	× 3.2–12.8	(Karpiński, et al. 2025b)	0.12	Low
	4	32-64	× 8-16	(Garratt et al. 2021)	0.064-0.128	Very low/Low
	32	256	× 8	(Tagliaferri et al. 2024)	**0.512**	Moderate
	4-8	32-128	× 4-32	(Shepherd et al. 2018)	0.064-**0.256**	Very low/ Moderate
C. albicans	1.95-3.9	7.5-10	× 1.9-5.1	(Karpiński et al. 2024)	0.019	Very low

Interpretation of the Karpinski Adaptation Index: KAI ≤ 0.1: very low risk of clinical resistance; 0.1 < KAI ≤ 0.2: low risk of clinical resistance; 0.2 < KAI ≤ 0.8: moderate risk of clinical resistance; 0.8 < KAI < 1.0: high risk of clinical resistance; KAI ≥ 1.0: very high risk of clinical resistance (Karpiński 2024).

7. Precautions and application of OCT

OCT meets the criteria for selecting antimicrobial products in the wound healing process, namely:

- it has broad-spectrum antimicrobial effectiveness and a fast action time,
- it has the ability to destroy biofilm,
- it has tissue tolerance, lacks cytotoxicity and carcinogenicity,
- it can be combined with surfactants and specialized dressings,
- it does not lead to the development of resistance,
- it is not inactivated by protein loads and pH changes (Kramer et al. 2018).

OCT-based products are recommended for wound prevention and treatment. Combinations such as 0.1% OCT + 2% PE or 0.05% OCT + ethylhexylglycerin are approved. Contraindications for using OCT products include: peritoneal lavage, fistulas, and other structures from which the applied substance cannot be thoroughly rinsed; use in the extraperitoneal space; use on hyaline cartilage and central nervous system structures; and allergy. OCT rarely causes side effects. Documented effects include blistering, necrosis, and scarring in

newborns (Biermann et al. 2016), contact dermatitis, and swelling (Calow et al. 2009; Biermann et al. 2016). The use of OCT without drainage may lead to persistent edematous changes, inflammatory reactions, and necrosis (Eigner et al. 2023).

According to the International Consensus Document "Use of Wound Antiseptics in Practice" from 2023 (Nair et al. 2023), guidelines of Polish Wound Management Association (Sopata et al. 2023) and the German Consensus on Wound Antisepsis (Kramer et al. 2018), OCT is the first-choice antiseptic for critically colonized wounds, infection-prone wounds, burns, wounds colonized by MDR pathogens or infected wounds, and for surgical site infections (SSI) prevention. OCT is also used in umbilical stump care (Mivšek et al. 2017), treatment of skin and mucosal fungal infections (Novakov Mikić and Stojic 2015) and bacterial vaginosis (Swidsinski et al. 2015). OCT inhibits dental plaque formation and is used in treating oral inflammation and periodontitis. Thus, it is an effective alternative to chlorhexidine and other contemporary mouthwashes (Grover et al. 2021; Rath et al. 2024). However, all antiseptics, like antibiotics, particularly when used for long periods, may lead to oral and intestinal dysbiosis (Amaral et al. 2023; Brookes et al. 2023; Contaldo et al. 2023). It is important for future studies to investigate the long-term influence of antiseptics, including OCT on host microbiota and its implications for antimicrobial stewardship.

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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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