Investigating Adaptation of *Staphylococcus aureus* to Hand Sanitisers and Subsequent Antibiotic Co-selection via Experimental Evolution

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfilment of the requirements for the degree of
B.Sc. in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Abstract

One of the most notable results of the COVID-19 pandemic is the escalation in the use of hand sanitisers as personal hygiene products as well as in healthcare settings. Besides devising effective infection control strategies, understanding how bacterial strains adapt to these agents is crucial, especially due to the possibility of an unwanted evolution of antibiotic-resistant strains. This study explored how *Staphylococcus aureus* responds to some common hand sanitiser formulations. We investigated microbial tolerance acquisition and potential antibiotic co-selection through experimental evolution. *S. aureus* was exposed to escalating sub-inhibitory hand sanitiser concentrations of Hexisol® (0.5% chlorhexidine gluconate & 70% isopropyl alcohol), Sepnil® (70% ethanol, carbomer, glycerin, polyethylene glycol, TEA, aqua, and perfume), and 70% Ethanol. We compared the Minimum Inhibitory Concentrations (MIC) between evolved and parent strains, performed antibiotic susceptibility testing, assessed fitness using a growth curve assay, and subcultured the evolved strains in sanitiser-free broth for 10 days to assess the stability of the acquired tolerance. Hexisol® showed a 4-fold increase in MIC against the adapted strain, co-selection for macrolide and β-lactam antibiotics, and increased susceptibility to other antibiotics. The growth capacity of the adapted strain was significantly decreased. Tolerance to Hexisol® remained stable, but antibiotic co-selection reversed after 10 days. 70% ethanol and Sepnil® showed similar results. The MIC of these hand sanitisers against the adapted strain did not significantly increase; however, it exceeded the recommended dose. No antibiotic co-selection occurred, but susceptibility to certain antibiotics increased. The growth capacity of the adapted strain was significantly decreased. Bacterial tolerance to 70% ethanol and Sepnil® remained stable after 10 subcultures. The findings shed light on the emergence of potential public health hazards resulting from microbial adaptation to hand sanitisers, emphasizing the need for reevaluation of their long-term impact on antibiotic resistance and infection control strategies.

Keywords: Hand Sanitiser; Bacterial Adaptation; Antibiotic Co-selection, Chlorhexidine

Dedication

To our friends and families, for their unwavering support,

and to everyone committed to scientific progress.

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List of Acronyms

AMR: Antimicrobial Resistance

AST: Antibiotic Susceptibility Test

CHX: Chlorhexidine

HGT: Horizontal Gene Transfer

MBC: Minimum Bactericidal Concentration

MFS: Major Facilitator Superfamily

MIC: Minimum Inhibitory Concentration

MRSA: Methicillin-Resistant *Staphylococcus aureus*

WHO: World Health Organisation

Chapter 1

Introduction

1.1 Background

The strategic use of biocides is a vital defence in the never-ending effort to protect public health against infectious pathogens. However, even the seemingly simple use of hand sanitisers as a frontline tool has come under scrutiny as antimicrobial resistance (AMR) continues to increase. The present review explores the complex interactions that occur between drug-resistant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA), and biocides, specifically focusing on hand sanitisers.

A common item in our daily lives, hand sanitisers are essential for stopping the spread of pathogens. However, the rising incidence of AMR, especially in the robust ESKAPE organisms, has brought to light possible connections between biocides and antibiotic resistance, leading to a reconsideration of their application. In this situation, *Staphylococcus aureus* stands out as a major threat that requires extra attention.

A major threat to public health is *S. aureus*, a clinically significant bacterium, as MRSA infections are on the rise globally. Increased resistance to agents such as chlorhexidine (CHX), which indicates biocide tolerance, is a critical factor in the rise of MRSA. Biocide resistance genes are closely associated with this resistance, suggesting possible genetic links between antibiotic resistance and biocide tolerance.

Efflux mechanisms, specifically those belonging to Major Facilitator Superfamily (MFS), are essential for reducing vulnerability to biocides, such as those in hand sanitisers. Reduction in susceptibility to CHX has been linked to the *qacA* gene, which is an important component of this genetic arsenal. Complex interactions among mobile genetic elements, antibiotic resistance

genes, and *qac* genes in the presence of various difficulties comprehending the dynamics of resistance transmission.

Although there is evidence of co- and cross-resistance between antibiotics and biocides, more research is necessary because of the complexity of this interaction. A comprehensive, all-encompassing approach to fighting AMR is even more important now that we know how important hand sanitisers are in our defence against infectious threats.

1.2 Significance of Disinfection in the Context of Multidrug Resistance

At present, the growing danger of AMR has attracted considerable attention in the field of global health. A global public health priority of the highest importance is effectively managing its emergence and subsequent proliferation. The 2015 global action plan on AMR published by the World Health Organisation strongly encourages careful consumption of antimicrobial agents, discourages their unnecessary use, and limits the spread of infections by implementing strong preventive, sanitary, and hygienic measures. Conscious use of disinfection has been revealed as a crucial element in the complex strategy to prevent the spread of pathogens resistant to multiple drugs, highlighting its important role in this joint effort (Exner et al., 2017; Gebel et al., 2013).

1.3 Hand Sanitisers: Composition and Mode of Action

Dispensable in liquid, gel, or foam forms, hand sanitiser is a biocidal agent that reduces the number of microorganisms on surfaces. Particularly considering the COVID-19 pandemic, the post-pandemic spike in hand sanitiser use is an essential public health measure aimed at reducing the transmission of infectious diseases (Lopez et al., 2022). Significant statistical data supports this upswing; Saha et al. (2021) reported a striking increase in hand sanitiser sales from February to March 2020 compared with the same period in the previous year, with figures ranging from 300% to 470%. In addition, a separate study by Lee et al. (2022) found that hand

sanitiser sales increased by an astounding 470% in the first week of March 2020. In particular, alcohol-based hand sanitisers have gained widespread recognition as an effective way to reduce the spread of viruses (Lopez et al., 2022). Nonetheless, it is crucial to recognise that the initial wave of hand sanitiser adoption might have been spurred by fear and a lack of unbiased information about how best to use them (Saha et al., 2021). Strong support for strict hygiene practises as a preventative measure against COVID-19 from scientists, doctors, and government officials is responsible for the noticeable increase in the use of hand sanitiser (Mahmood et al., 2020).

1.3.1 Composition of Commercially Available Hand Sanitisers

Guidelines for the production of hand sanitisers by local manufacturers have been released by the World Health Organisation (WHO). In particular, the World Health Organisation has recommended two formulations for production on a smaller scale: one that uses 96% ethanol and the other that uses 99.8% isopropyl alcohol. Within the household or local production context, the WHO recommends a final product concentration of 80% v/v ethanol, 1.45% v/v glycerol and 0.12% v/v hydrogen peroxide for formulation A and 75% v/v isopropyl alcohol, 0.12% v/v, hydrogen peroxide, and 1.45% v/v glycerol for formulation B (Mahmood et al., 2020). Neither of the formulations recommended by the WHO contained any added non-volatile active ingredients that could provide sustained efficacy, despite the WHO's recommendation for hand sanitisers with sustained activity (Kampf et al., 2017). On the other hand, many hand rubs sold in stores contain ingredients such as 0.5 or 1% CHX (additional non-volatile active ingredient), frequently found in mixtures containing 61% ethanol or 70% isopropanol. Consumers of these products are likely to experience a sustained effect from the entire formulation.

1.3.2 Mode of Action: Hand Sanitisers Components

Contrary to most antibiotics, only some biocides target a single site within the microbe's cell. Biocides, such as hand sanitisers, work by disrupting hydrogen bonds, ionic interactions and chemical reactions involving oxidants and electrophiles to affect multiple sites within bacteria in a non-discriminatory manner. The exact mechanisms underlying these processes are still not fully understood (Coombs et al., 2023).

1.3.2.1 Alcohol

The most commonly used compounds from the alcohol group are ethanol and isopropanol. Elekhnawy et al. (2020) reported that although they do not affect spores, they exhibit swift and broad-spectrum activity against bacteria, viruses, and fungi. DNA precipitation by the removal of hydrogen bonds and protein denaturation via hydrogen disruption are the primary mechanisms by which alcohol acts. This then disrupts the structure and function of the membrane, causing cellular components to leak out, interfering with cell metabolism and function, ultimately leading to cell lysis (McDonnell & Russell, 1999).

1.3.2.2 Chlorhexidine

In its water-soluble gluconate form, CHX is frequently used at different concentrations, from 0.5% to 4% (Horner et al., 2012). CHX is a biguanide and a cationic antimicrobial (Gilbert & Moore, 2005). Although it is ineffective against spores, it has bactericidal and fungicidal properties and exhibits activity against some viruses. According to Hugo & Longworth (2011), CHX works by attaching itself to the negatively charged bacterial cell wall and altering its osmotic balance. It inhibits cytoplasmic membrane function, affecting enzymes bound to membranes, causing coagulation and cytoplasmic precipitation, and allowing the release of intracellular components. In addition, it acts as an inhibitor of membrane-bound and soluble

ATPase and net K+ uptake. It also has the ability to collapse the membrane potential and inactivate ATPase.

1.3.2.3 Hydrogen Peroxide

One of the most potent oxidizing agents is hydrogen peroxide, which can effectively destroy bacteria, yeast, bacterial spores, and viruses. The mode of action of hydrogen peroxide is forming free hydroxyl radicals (·OH) which are highly reactive molecules with strong oxidizing abilities. According to Vantansever et al. (2013), these radicals can interact with essential cellular constituents such as membrane lipids, ribosomes, proteins, enzymes, and DNA, ultimately resulting in their degradation.

1.3.2.4 Glycerol

Glycerol lacks antimicrobial qualities, but it is beneficial for the skin barrier. Glycerol is a humectant that helps to keep the skin hydrated (Fluhr et al., 2008). It pulls in water from the environment and adhere them to the skin, thereby enhancing moisture levels. Moreover, it promotes the development of tight junctions between skin cells, strengthening the integrity of the stratum corneum, the outermost layer of the skin. The skin's natural defenses against external infections and irritants are thereby reinforced (Fluhr et al., 2008). However, it was discovered that glycerin was among the associated contents that interfered the most; its three -OH groups overlapped with the ethanol spectrum (Littlejohn et al., 1991). Thus, elevated glycerin concentrations exceeding 1.45% may reduce the antimicrobial effectiveness of alcohols (Menegueti et al., 2019).

1.4 Determinants Impacting the Effectiveness of Biocides

A wide range of internal and external factors can have an impact on the effectiveness of biocides. Extrinsic factors depend on the surroundings when the biocide is applied, whereas inherent factors are related to the particular biocidal agent and its application. From an intrinsic

perspective, the concentration of the biocidal material and the time of contact of the compound and microbe are critical factors (Scientific Committee on Emerging and Newly-Identified Health Risks, 2007). Biocides are usually used at concentrations higher than those that are fatal to microorganisms. However, several factors may prevent the biocidal agent from reaching its maximum concentration when it comes into contact with the microorganism. For example, dust particles may hinder the biocidal agent's ability to interact with cells (Russell & McDonnell, 2000). Critical external factors include pH and temperature (Scientific Committee on Emerging and Newly-Identified Health Risks, 2007). As a result, misusing biocides can reduce their effectiveness and expose bacteria to concentrations of these compounds that are either non-lethal or subinhibitory.

1.5 Bacterial Adaptation to Biocides

1.5.1 Terminologies Related to Biocide Adaptation

It is crucial to understand that biocide efficacy lacks standardised methods or definitions, in contrast to antibiotic resistance, which benefits from established methodologies and definitions for assessing clinical therapeutic efficacy. Biocide susceptibility testing is often conducted using techniques developed for antibiotic research. Furthermore, several terms are used concerning biocides, including "decreased susceptibility," "reduced susceptibility," "tolerance," and "resistance," (Maillard, 2018). This variety highlights the need for more agreement among scientists, which adds to the difficulty in understanding how bacteria become resistant to biocides. According to the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), tolerance is indicated by an elevated minimal inhibitory concentration (MIC) and decreased susceptibility to an antimicrobial agent. In contrast, resistance refers to a situation in which the strain is not inhibited or eliminated by the concentrations usually recommended for use by the manufacturers.

1.5.2 Bacterial Adaptation to Hand Sanitiser Components

The discovery that bacteria can adapt to biocides is not new; it was first reported more than 50 years ago (Russell, 2004). Despite advances in our understanding, the mechanisms that bacteria use to reduce their susceptibility to biocides remain largely unknown. Biocides use numerous target sites within microbial cells to exert their effects. Therefore, compared with the common resistance mechanisms observed in therapeutic antimicrobials (van Hoek et al., 2011), cases of decreased susceptibility resulting from target site alteration or metabolic process bypass are rare. Bacteria usually show decreased biocide susceptibility by reducing the biocide concentration until it no longer damages the bacterial cells. The ability of bacteria to repair damage is one example of this ability (Scientific Committee on Emerging and Newly-Identified Health Risks, 2007). In bacteria, several mechanisms for decreased susceptibility to biocides have been identified; these mechanisms can be intrinsic or acquired. Acquired resistance is the outcome of acquiring genetic elements (such as plasmids or transposons) or mutations; intrinsic resistance is an innate property of the cell.

The inherent qualities of the biocide itself and the traits of the organism have a major impact on how bacteria react to particular biocides and the type of resistance they develop. In this experiment, we will specifically address microbial tolerance to the common components of hand sanitisers, which are hydrogen peroxide, CHX, and alcohol.

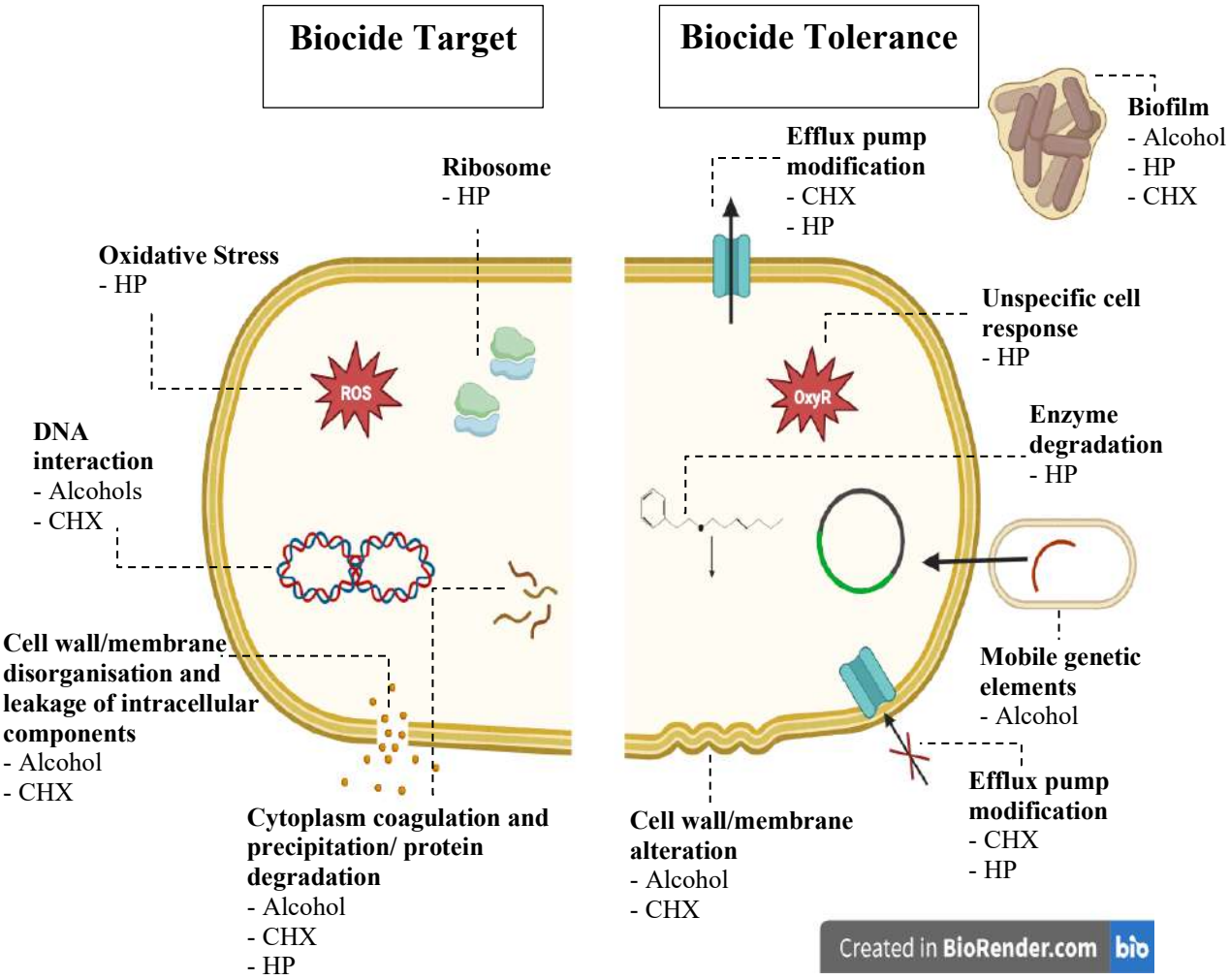


Figure 1: A simplified illustration of biocide target sites and biocide tolerance mechanisms in bacterial cell. (reproduced from Merchel Piovesan Pereira & Tagkopoulos, 2019). CHX= chlorhexidine, HP= hydrogen peroxide, ROS= reactive oxygen species, OxyR= bacterial peroxide sensor. Created with BioRender.com.

1.5.2.1 Bacterial Adaptation to Alcohol

Bacterial adaptation to alcohols includes horizontal gene transfer, transformation, transduction, and core genome mutations, especially in the chromosomal nucleotide position on the RNA polymerase *rpoB* gene β subunit. For example, a noteworthy finding by Pidot et al. (2018) was that hospital-derived *Enterococcus* faecium isolates obtained after 2010 had a ten-fold higher

tolerance to alcohol than isolates obtained before 2004. More than 400 shared nucleotide mutations were found in correlation with this shift. Furthermore, their investigation demonstrated that the alcohol-tolerant *Enterococcus faecium* strains continued to be able to infect mice and colonise their intestines even after being disinfected with 70% isopropanol. The critical role that horizontal gene transfer (HGT) plays in granting resistance to stressors was highlighted by another study. According to the study, *E. coli* has the ability to acquire ethanol tolerance over several generations and then pass on this trait via HGT. Through transduction, wild strains were able to acquire 45% v/v tolerance and up to 55% v/v tolerance through transformation. Nonetheless, Pidot et al. (2018) reported no notable changes in Minimum Inhibitory Concentrations (MIC).

Alcohol tolerance in bacteria can also be obtained by alterations of the cell membrane. Ethanol has the ability to alter the proportion of unsaturated fatty acids (USFA) to saturated fatty acids (SFA) in the cell membrane. In *V. parahaemolyticus*, exposure to ethanol resulted in a lower SFA to USFA ratio. This shift in the ratio is probably related to improved ethanol tolerance and modifications in cell permeability (Chiang et al., 2008). Other bacterial species, such as *Z. mobilis* and *E. coli*, have also been shown to exhibit similar relationships between ethanol tolerance and fatty acid composition; these bacteria adapt by altering the composition of fatty acid of their membranes. Increasing the length of fatty acid chains from 16 to 18 carbons is a crucial adaptation. This strengthens the hydrophobic barrier by increasing the quantity of CH_2 units (Ingram, 1989). Adaptive changes also include lowering the lipid-to-protein ratio, which reduces the number of lipid patches accessible on the surface of the membrane for passive leakage, and retaining phospholipids with the tightest associations with membrane proteins. In *S. aureus*, ethanol causes an overall upregulation of genes linked to fatty acid metabolism, nucleotide synthesis, and energy metabolism (Korem et al., 2010).

Bacteria exposed to ethanol (alcohol) exhibit elevated expression of several genes encoding surface proteins, such as *sdrCDE, icaAD, mapW, spa, and clfB* (Luther et al., 2015)*;* these proteins may be necessary for cell attachment. In *S. aureus* biofilm, the expression of these proteins can be observed. In addition, in the presence of alcohol, genes that improve bacterial survival within the biofilm are upregulated (Resch et al., 2005).

1.5.2.2 Bacterial Adaptation to Chlorhexidine

The term "disinfectant failure" was coined in the early 1970s for CHX preparations. This was brought on by some Gram-negative species showing resistance to the disinfection treatment, such as *Pseudomonas* spp. or *Klebsiella* spp. (Rutala & Weber, 2015). Many outbreaks involving various species of bacteria, such *P. aeruginosa, S. marcescens, Pseudomonas* spp., *B. cepacia, A. xylosoxidans*, and *R. picketti* have been linked to contaminated CHX solutions, usually at 0.05% (Weber et al., 2007). These studies demonstrate that microorganisms have a strong capacity to adapt to CHX. According to Hassan et al. (2013), the three main mechanisms responsible for this adaptability are elevated efflux pump activity, modified membrane permeability, and the development of bacterial biofilms.

One mechanism includes the increased expression of RND efflux pumps such as AcrAB-TolC due to gene mutations such as *marA*, resulting in increased CHX resistance (Levy, 2002). The MIC of CHX were considerably higher in clinical isolates of *Acinetobacter baumannii* carrying the RND efflux pump-encoding genes *qacE. adeB*, and *adeJ* (Rajamohan et al., 2010). Moreover, elevated CHX MIC in *E. coli* biofilms was linked to the overexpression of SMR pumps, such as QacE, QacEΔ1, and EmrE (Srinivasan et al., 2009). Furthermore, according to Muñoz-Gallego et al. (2016), the presence of MFS pumps, QacA and QacB, in *Staphylococci* isolates was linked to decreased susceptibility to CHX. Mutations in the *efrA* and *efrB* genes have been observed in *Enterococcus*, altering the expression of the EfrAB efflux pump of the ABC family and the hydrophobicity of the bacterial surface (Kitagawa et al., 2016; Nasr et al.,

2018). Mutations in the *qacA, qacB, smr*, and *norA* genes have been found in *S. aureus* and MRSA cases (Hasanvand et al., 2015; Noguchi et al., 2006). For *P. aeruginosa*, mutations were observed in efflux pump genes such as *oprH-phoPQ* and *MexCD-OprJ* which were set off by the stress response factor *AlgU*; genes that make proteins involved in electron transport, membrane transport, oxidative phosphorylation, and DNA repair were also downregulated (Sheng et al., 2009; Vijayakumar et al., 2018).

Changes in porin profiles are another mechanism that affects the state of the outer membrane and increases the CHX MIC. This mechanism has been observed in *Pseudomonas stutzeri* (Tattawasart, Maillard, et al., 2000) and the bacterial surface of the adapted strain was more hydrophobic compared to the wild-type strain. Changes in porin expression were also observed in *E. Coli* cultures that had adapted to a CHX-containing medium, suggesting an adaptation response (Gregorchuk et al., 2021). Furthermore, *E. coli* susceptibility to CHX decreased upon the loss of MlaA, suggesting that MlaA is involved in the cellular uptake of CHX (Chong et al., 2015).

In addition, bacterial biofilm formation contributes to decreased susceptibility to CHX. Cationic antimicrobials, such as CHX, are less effective when bound by extracellular DNA (eDNA) in biofilms because of their negative charge (Gränicher et al., 2021). According to Gränicher et al. (2021) the formation of biofilms also upregulates multidrug efflux pumps, which increases resistance to antimicrobial agents. Extracellular polysaccharide abundance functions as a barrier, preventing CHX from penetrating deep biofilm layers and possibly developing antimicrobial resistance (Maillard, 2018).

To effectively combat CHX-resistant bacteria in clinical settings, it is vital to understand these mechanisms.

1.5.2.3 Bacterial Adaptation to Hydrogen Peroxide

The *katA* gene specifically facilitates the activity of enzymes such as catalase and peroxidase, which are necessary for bacterial adaptation to hydrogen peroxide. The evidence that is currently available indicates that group A *Streptococcus* and *E.coli* can both acquire hydrogen peroxide tolerance in a brief amount of exposure time (Dukan & Touati, 1996; Henningham et al., 2015).Henningham et al. (2015) observed that group A Streptococcus produces enzymes that degrade hydrogen peroxide, such as NoxA and AphC oxidase, while another study by Demple (1996) concluded that catalase and superoxide dismutase increase the resistance of Escherichia coli to hydrogen peroxide and superoxide. Bacteria use various strategies to reduce the oxidative stress caused by hydrogen peroxide.

Certain transcriptional regulators, such as SoxR and OxyR, are essential for the protection of *E. coli* against oxidative stress. Proteins that aid in detoxification, DNA repair, protection systems, and other processes are also produced by these regulators (Zheng et al., 2001). hydrogen peroxide activates OxyR, creating an intramolecular disulfide bond and positively regulating the enzymes that scavenge hydrogen peroxide. This response is self-regulated by a feedback loop that includes enzymes such as glutaredoxin I (Zheng et al., 1998). Furthermore, Ezraty et al. (2013) stated that Suf protein induction helps to supply the necessary iron–sulfur clusters for critical biochemical pathways that are interfered with by hydrogen peroxide stress. Moreover, by acquiring unincorporated iron, the activation of Dps, a ferritin-class protein, aids in suppressing hydrogen peroxide-induced DNA damage (Ezraty et al., 2013). Exposure to hydrogen peroxide can also induce thick biofilm formations that help protect the bacterial cells (Rozman et al., 2021).

1.5.2.4 Summary

The phenomenon of bacterial adaptation to biocides is well-researched and involves mechanisms that reduce the harmful effects of these agents. Genetic mutations and mobile genetic elements can be acquired or intrinsic, resulting in decreased biocide uptake. The three main components of biocide tolerance are flux pumps, permeability modifications, and biofilm formation. Responses to particular biocides, such as alcohol, hydrogen peroxide, and CHX, differ and can involve genetic mutations that affect permeability and efflux pumps. Comprehending these mechanisms is imperative for managing bacteria resistant to biocide within healthcare settings.

1.6 Influences of Biocide Adaptation on Bacterial Fitness

Bacterial adaptation to biocides and antibiotics often involves non-specific metabolic costs, which could have adverse effects on biological fitness, such as slower growth (Deptuła & Gospodarek, 2010; Gilbert & McBain, 2003). Broad-spectrum efflux pumps are one prominent example; they require cellular energy and indiscriminately remove vital metabolic components from microbial cells Sonbol et al. (2019). Another example is the presence of plasmids resistant to biocides and antibiotics, whose stability and fitness cost require selection pressures to be maintained (Gullberg et al., 2014). Bacterial fitness costs can be quickly identified in laboratory settings by looking for decrease in growth capacity or colony size (Gullberg et al., 2014). Elekhnawy et al. (2020) noted that co-selection is generally a lesser burden and that prolonged exposure to biocides may induce compensatory adaptations that increase bacterial fitness.

1.7 Antibiotic Resistance Mechanisms

To protect themselves from antibiotics, bacteria use a variety of resistance mechanisms (Tenover, 2006). Typical resistance mechanisms include (a) decreased absorption, resulting

from a decrease in membrane permeability, which restricts the entry of antibiotics into cells (e.g., tetracycline and quinolone resistance); (b) enzymatic inhibition or inactivation of the antibiotic (e.g., β-lactam resistance via β-lactamases); (c) rapid expulsion of the antibiotic from the cell (e.g., tetracycline and macrolide resistance); (d) modifications to the target site, accomplished by mutations in the target receptor (e.g., oxacillin and methicillin resistance via *mecA* gene mutation); and (e) acquisition of an alternate metabolic pathway not impacted by the antibiotics (e.g. sulfonamide resistance). Because antimicrobial agents usually have particular targets within the cell, acquiring any of these mechanisms can confer resistance to the antimicrobial agent or several compounds in the same group (van Hoek et al., 2011).

1.7.1 Biocide-Induced Antibiotic Resistance

Particularly in the context of antibiotics, antimicrobial resistance has attracted considerable attention from the scientific and medical community (Czaplewski et al., 2016; Sakudo et al., 2019). Comparatively, less attention has been paid to disinfectants despite their widespread use, particularly in clinical settings. The correlation between the use of biocides and the development of antibiotic resistance in specific bacterial species is still under investigation (Chen et al., 2021). According to Maillard (2018), exposure to biocidal agents can cause a stress response that involves the activation of global gene regulators and, in turn, the expression of non-specific mechanisms that allow bacteria to survive. The use of biocides and antibiotic resistance has generated much discussion, but the evidence supporting this relationship has been inconsistent; some studies support the relationship, whereas others find no co-resistance (Maillard, 2018). Notably, biocides such as CHX diacetate and antibiotics are important antibacterial agents. There have been suggestions that the mechanisms of action of biocides and antibiotics may be similar (Avrain et al., 2007; Thomas et al., 2005). Studies conducted in laboratories have documented cases in which antibiotic and biocide resistance has been co-

selected. Bacteria resistant to one class of antibiotics may also become resistant to other antibiotics or less susceptible to low-concentration biocides. Similarly, co-selection of antibiotic resistance can also arise from the insensitivity of bacteria to biocides at low concentrations (Langsrud et al., 2003). When co-selection of resistance between exposure to biocide and antibiotics was noted, possible common mechanisms of resistance included changes in bacterial metabolism (Webber et al., 2008), overexpression of efflux (Grande Burgos et al., 2016; Maillard et al., 2013), and modifications in cell wall permeability (Tattawasart, Hann, et al., 2000; Tattawasart, Maillard, et al., 2000). Multidrug-resistant and multiple biocide-resistant pathogens will emerge in outbreaks because biocides are widely used in different contexts and their use is less regulated than antibiotics. The Scientific Committee on Emerging and Newly Identified Health Risks (Scientific Committee on Emerging and Newly-Identified Health Risks, 2007; Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2010) and the Scientific Committee on Consumer Safety (Davison & Maillard, 2010) have both released reports that have prompted the European Commission to express concern. The regulations governing the commercialization of biocidal products in the European market, known as the Biocidal Product Regulation (Pedrouzo et al., 2009), now recognise the possibility of bacterial resistance and cross-resistance after biocide application.

1.7.2 Fundamental Elements of Co-Selection Mechanism

Through co-selection mechanisms, the overuse and improper application of biocides can reduce the sensitivity of target organisms to essential antimicrobials (Rozman et al., 2021). The terms "co-resistance" and "cross-resistance," though they have different meanings, are frequently used to describe the adaptive responses of microbes that exhibit resistance or decreased susceptibility to biocidal or antimicrobial agents (Buffet-Bataillon et al., 2011; Elekhnawy et al., 2020). First, the phenomenon where different mechanisms of tolerance or

resistance are encoded by the same mobile genetic element is denoted as co-resistance. Second, cross-resistance in a given microorganism indicates that the same mechanism is responsible for different antibiotic or biocide tolerances.

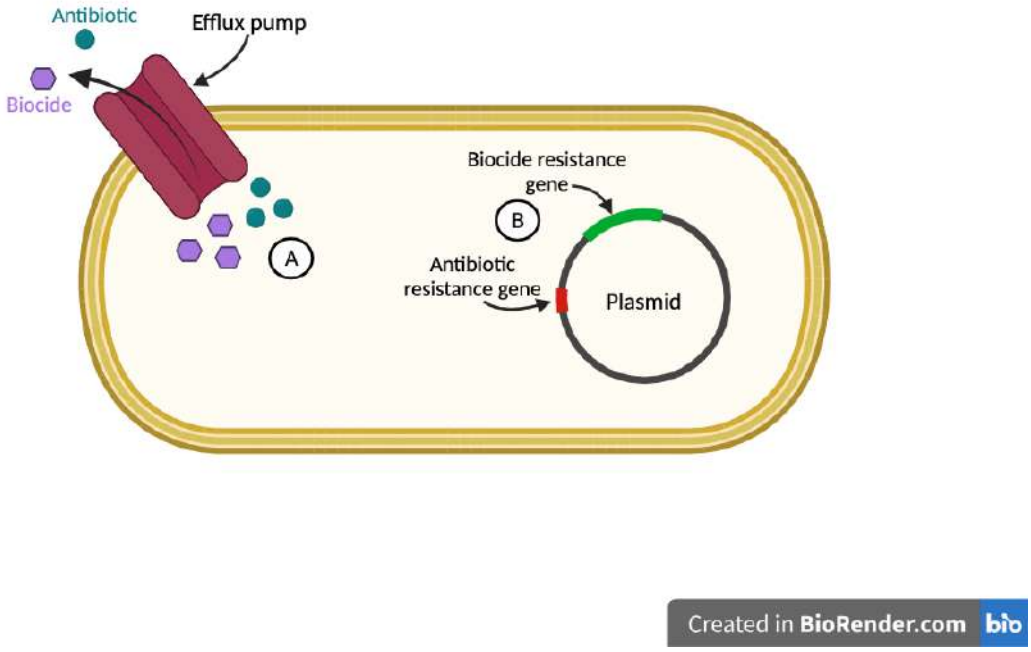


Figure 2: Co-selection mechanisms in bacteria. A: Cross-resistance; B: Co-resistance. (adapted from Elekhnawy et al., 2020). Created with BioRender.com.

1.7.3 Studies Demonstrating Biocide-Induced Antibiotic Resistance

The literature primarily focuses on phenolics, biguanides such as CHX, and Quaternary Ammonium Compounds (QACs). By comparison, the amount of information on metals, fixatives such as formaldehyde, peroxygens such as hydrogen peroxide, alcohols, and iodine is relatively less. There are two primary categories of studies in the body of current literature. One side of the research focuses on tests in which bacterial strains are exposed over several generations to progressively higher sub-inhibitory concentrations of biocides to induce an adaptive response that increases biocide tolerance (experimental evolution). A less-explored

area is the analysis of bacterial isolates obtained from a range of environmental samples, including lakes, food processing zones, hospital surfaces, mining sites, wastewater treatment plants, and agricultural areas (Coombs et al., 2023). Recurrent exposure of bacteria to subinhibitory levels of biocidal agents increases the likelihood of developing biocide-induced antibiotic resistance, according to available data. According to Gilbert & McBain (2003), the concentration of biocides in practical applications is usually present as a continuous gradient that ranges from zero to the in-use concentration, making this probable.

1.7.3.1 Alcohol-Induced Antibiotic Resistance

Alcohols do not appear to cause antibiotic cross-resistance, according to the available literature. According to Merchel Piovesan Pereira et al.'s study from 2021, bacteria exposed to sublethal concentrations of isopropanol and isopropanol (2.5% v/v and 4.25%, respectively) for approximately 500 generations did not show signs of antibiotic cross-resistance. Furthermore, an extensive examination of different antibiotics and biocides determined that alcohols showed the least tolerance development in clinically isolated strains of all the biocides examined (Shan et al., 2018).

1.7.3.2 Chlorhexidine-Induced Antibiotic Resistance

An ongoing discussion in the scientific community has focused on the possibility of cross-resistance between antibiotics and CHX. According to Russell (2010) and Russell et al. (1998), although the frequent use of CHX has not induced in the development of clinically significant antibiotic resistance, such resistance has increased, especially in clinical healthcare settings where high selection pressure is present. Divergent viewpoints on this matter have been presented in a number of studies. Some studies could not prove a relationship between antibiotic susceptibility and CHX, for example, in genetically different *Salmonella* spp. and *B. cepacia* complex isolates from turkey farms (Beier et al., 2011). In contrast, other studies have

shown that resistance to antibiotics and antiseptics such as CHX is positively correlated in certain bacterial species, such as *S. marcescens* and *Alcaligenes* spp. (Maris, 1991).

Two main modes of action explain cross-resistance: alteration of theouter membrane concerning the lipopolysaccharide (LPS) profile and electrostatic activity and overexpression or activation of multidrug efflux pumps. The relevance of alterations in the outer membrane's LPS profile and electrostatic activity has been highlighted by interesting findings regarding cross-resistance between CHX and colistin in carbapenem-resistant K. pneumoniae infections (Falagas et al., 2014; Wand et al., 2017). This occurs because both CHX and colistin bind to the negatively charged LPS; thus, intracellular components leak out. Cross-resistance between CHX and colistin is believed to be caused by the increased expression of the *pmrK* gene and decreased LPS anionic charge (H. Lee et al., 2004; Tamayo et al., 2005).

Because of their broad substrate selectivity, multidrug efflux pumps are essential mediators of cross-resistance when activated and/or overexpressed. Among the chromosomal genes encoding MDR efflux pumps are *mdeA, mepA norC, norA, lmrS, sepA,* and *sdrM. QacA/B*, smr (qacC), qacG, qacH, and qacJfound in Staphylococci are examples of the numerous MDR genes carried on plasmids (Heir et al., 1998; Jonas et al., 2001; Paulsen et al., 1995). This raises questions regarding the role of horizontal gene transfer in intra- and interspecies spread. The overuse of CHX is likely to stimulate the upregulation of MDR efflux pump genes through selective environmental pressure.

Furthermore, strains of *A. baumannii* with decreased sensitivity to CHX exhibit co-resistance to antibiotics and aminoglycosides, carbapenem, tetracycline, and ciprofloxacin (Fernández-

Cuenca et al., 2015). Moreover, research has demonstrated that healthcare personnel who use CHX-based soap have a higher relative risk of antibiotic-resistant *Staphylococcus epidermidis* strains, including those resistant to gentamicin and oxacillin, colonizing their hands (Cook et al., 2007); in the same study, a noticeably elevated relative risk of rifampicin resistance was observed in *Staphylococcus warneri* after exposure to CHX. Furthermore, nosocomial *S. aureus* isolate analyses showed that isolates positive for the *smr* and *qacA/B* genes had increased resistance rates, highlighting the significance of these genetic elements in multidrug resistance. Research conducted over several decades on multi-resistance plasmids in staphylococcal isolates revealed strong selective pressure affecting the organization and content of plasmids. In addition, resistance to CHX, as well as to several antibiotics and biocides, was introduced by the plasmid pSAJ1 derived from a strain of *S. aureus* that was resistant to both methicillin and gentamicin (McNeil et al., 2016; Wand et al., 2015).

These results show that although CHX-antibiotic resistance may not be commonplace at present, there is evidence that it might develop in specific bacterial species and environments.

1.7.3.3 Hydrogen Peroxide-Induced Antibiotic Resistance

Insufficient and inconsistent data regarding hydrogen peroxide's ability to cause antibiotic cross-resistance exist. A previous study found that some strains of *E. coli* were less susceptible to antibiotics in laboratory settings when exposed to low concentrations of hydrogen peroxide (Merchel Piovesan Pereira et al., 2021). In addition, Wesgate et al. (2016) found that prolonged exposure to low hydrogen peroxide levels caused "unstable resistance."

1.7.3.4 Summary

In conclusion, the effect of disinfectants on resistance has been largely disregarded, although antimicrobial resistance in antibiotics has drawn much attention. Most studies highlighting possible connections between antibiotic resistance and biocide exposure provide evidence for these connections in the literature. Biocide-induced resistance involves mechanisms of co- and cross-resistance. Most chemical disinfectant users do not anticipate that these biocidal agents could also contribute to antibiotic resistance. Triclosan is a well-known example. It was safe and effective for decades when used in antimicrobial soaps in US homes. However, the US Food and Drug Administration banned triclosan along with 19 active ingredients from disinfectants used by the public in 2016. In addition, it is recommended to review disinfectant groups, such as alcohol-based hand sanitisers. When it comes to reducing healthcare-associated infections, they are usually the first option that healthcare workers consider when cleaning their hands. There is no proof that additional biocidal ingredients in alcohol-based hand rubs, such as octenidine or CHX, benefit health. Therefore, it is necessary to evaluate how they are used. Hand rubs containing CHX will be the second largest source of total CHX exposure if they are regularly used for hand hygiene. Hand rubs containing CHX are not advised by the WHO, and their overall efficacy on dry hands is, at best, questionable.

1.4 *Staphylococcus aureus*: Clinical Significance, Biocide Adaptation, and Biocide-Induced Antibiotic Resistance

1.4.1 Clinical Significance of *Staphylococcus aureus*

An essential requirement for maintaining public health in an era characterized by the rise of antibiotic resistance and new infectious diseases is the efficient application of biocidal control measures. The complex relationship between biocide uses and the spread of AMR species—particularly the emergence of bacteria resistant to multiple drugs—is becoming increasingly

clear. The WHO action plan and the One Health strategy require active vigilance and monitoring of AMR species, but the issue of biocidal resistance is often overlooked. According to Meade et al. (2021), ESKAPE pathogens, including *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species, have demonstrated simultaneous resistance to biocides and pharmaceutical drugs. This increases the likelihood of increased patient mortality. *S. aureus* is one of these bacteria that is the focus of this investigation.

S. aureus is a significant pathogenic bacterium known for its involvement in a diverse range of diseases affecting the skin, soft tissues, as well as internal organs and endovascular sites. This bacterium is commonly present in both the skin and nasopharynx (JFOSTER, 2002). In normal circumstances, it forms part of the natural microflora on human skin and mucous membranes, usually without causing any harm. However, if it gains access to the bloodstream, it has the potential to lead to severe infections (Taylor & Unakal, 2023). Staphylococcus aureus has the capacity to instigate infections acquired within communities as well as those acquired within hospital settings. As a member of the Micrococcaceae family, it is characterized by its gram-positive nature. The genome of this bacterium is circular, encompassing approximately 2.8 Mb (2800 genes). Approximately 15–20% of the *S. aureus* genome consist of Mobile Genetic Elements (MGEs), collectively referred to as the mobilome. This category includes plasmids, bacteriophages, *S. aureus* pathogenicity islands (SaPIs), transposons, and the staphylococcal cassette chromosomes (SCC) (McCarthy et al., 2014). These elements can be gained or lost from genome via mechanisms of horizontal gene transfer (HGT), as outlined by Lindsay (2014). Infections attributed to methicillin-resistant strains of *S. aureus* have emerged as a major concern due to their extensive drug resistance capabilities.

1.4.2 Methicillin-Resistant *Staphylococcus aureus* (MRSA)

MRSA stands as a significant nosocomial pathogen, known for causing infections in healthcare settings. Each MRSA variant contains SCCmec, which hosts the *mecA* gene (Natalia et al., 2010). This gene provides resistance to methicillin and all antibiotics belonging to the β-lactam class . The SCCmec ranges from 21 to 67 kbp in size (Lim et al., 2012). The development of methicillin resistance in staphylococci arises from the integration of the SCCmec, a mobile genetic element that harbors the *mecA* gene along with various genes conferring antibiotic resistance. The *mecA* gene is responsible for encoding the penicillin-binding protein PBP2a, which exhibits a diminished affinity for β-lactam antibiotics. As of now, researchers have identified and scrutinized 14 main types of SCCmec (Mikhaylova et al., 2022). Community-associated MRSA (CA-MRSA) strains are known to commonly contain SCCmec IV, V, or VII elements. On the other hand, hospital-associated MRSA (HA-MRSA) strains predominantly carry the SCCmecI, II, III, VI, or VIII elements. These elements may also encompass supplementary resistance factors in addition to *mecA* (Ghaznavi-Rad et al., 2010). Additional resistance elements are commonly carried by plasmids, transposons, or incorporated sequences within the J regions of SCCmec (Ito et al., 2003). For example, within the J2 region, there exists a Tn554 transposon responsible for encoding erythromycin (*ermA*) and conferring resistance to streptomycin/spectinomycin (*aad9*) genes. Additionally, SCCmec elements may harbor genes conferring resistance against various classes of antibiotics, including macrolides, aminoglycosides, tetracyclines, lincosamides, and streptogramin B ("Classification of Staphylococcal Cassette Chromosome Mec (SCC Mec): Guidelines for Reporting Novel SCC Mec Elements," 2009).

1.4.3 Adaptation of *Staphylococcus aureus* to Biocide

According to Conceição et al. (2016), MRSA strains have developed greater resistance to biocides such as CHX and biocide resistance genes, such as *qac* genes, are associated with this

increased tolerance. The placement of such genes on mobile genetic elements (such as pSK1) raises the possibility of a relationship between antibiotic resistance and biocide tolerance. In addition, fluoroquinolones and biocides can be eliminated by the *norA*-encoded efflux pump (chromosome-based) belonging to the Major Facilitator Superfamily (DeMarco et al., 2007).

Efflux is the primary mechanism by which *S. aureus* develops decreased susceptibility to CHX. Clinical isolates of *S. aureus* with decreased susceptibility to CHX have been reported in recent years; these isolates are primarily attributed to the presence of the closely related plasmid-based genes *qacA* and *qacB* (do Vale et al., 2019). These genes encode multidrug efflux pumps reliant on proton-motive force, which play a role in exporting a variety of antiseptic compounds, including chlorhexidine (Smith et al., 2007). Instances of *qacA/B* genes have been identified in healthcare-associated MRSA (HA-MRSA) isolates worldwide (Smith et al., 2007). Of the known biocide resistance genes, staphylococci's susceptibility to CHX is notably reduced when *qacA* is involved. However, staphylococci may appear susceptible even in the presence of *qacA*, and the presence of *qacA* does not guarantee that an isolate will exhibit phenotypic resistance to CHX, demonstrating the complexity of this phenomenon (Horner et al., 2012). It is unclear how phenotypically decreased CHX susceptibility and the presence of CHX resistance genes, such as *qacA*, are related. An additional approach to exploring this variability would be to quantify the RNA expression of genes resistant to CHX during exposure to the agent.

1.4.4 Biocide-Induced Antibiotic Resistance in *Staphylococcus aureus*

Co-resistance plays an important role here, resulting from the coexistence of genes that confer resistance to antimicrobials and CHX resistance genes. The coexistence of *qacA/B* genes linked to β-lactamase resistance mediated by *blaZ* in coagulase-negative staphylococci is an example of this complex genetic interplay (Sheng et al., 2009). Interestingly, *qacA/B* is primarily found on the pSK1 family of multi-resistance *S. aureus* plasmids, which confer resistance against

trimethoprim and aminoglycosides, as well as on pSK57, a β-lactamase/heavy metal resistance plasmid (Russell, 1997). According to a study conducted by Johnson et al. in 2015, identified a plasmid (pC02) linked to reduced susceptibility of chlorhexidine (housed *qacA* gene). Upon detailed sequence analysis, pC02 was found to contain genes conferring resistance to β-lactams, antiseptics, and erythromycin, cadmium (Johnson et al., 2015). There have been documented instances of a genetic linkage between *qac* genes and genes that provide resistance to erythromycin, aminoglycosides, and trimethoprim on the same plasmid found in staphylococci (Noguchi et al., 2006). The presence of *qacA/B* genes has been observed in MRSA strains harboring SCCmec (mobile genomic element) which frequently carry extra genes that confer antibiotic resistance which makes treatment more challenging ("Classification of Staphylococcal Cassette Chromosome Mec (SCC Mec): Guidelines for Reporting Novel SCC Mec Elements," 2009). The presence of *mecA* on a mobile genetic element acts as a carrier for the transfer of genes between staphylococci bacteria, allowing for the incorporation of extra antibiotic-resistant genes from plasmids (Hanssen & Ericson Sollid, 2006). As some biocide tolerance can be facilitated by plasmids, there is a concern that exposure to biocides could potentially promote the dissemination of antibiotic resistance. This would occur through the selection and dispersal of plasmids that mediate resistance to both biocides and antibiotics.

According to multiple studies, MRSA is less sensitive to CHX than MSSA (Smith et al., 2007; Suller & Russell, 1999; Wootton et al., 2009). Additionally, earlier research indicates that *qacA/B* is more commonly found in MRSA strains compared to MSSA (Lu et al., 2015). An observed link between *qac* genes and resistance to multiple antibiotics, as supported by clinical findings, indicates that strains containing both biocide and antibiotic resistance genes may have a competitive advantage (Zhang et al., 2011). This finding is especially concerning because CHX is frequently used to decolonize MRSA. More research is needed to determine the effects

of using lower concentrations of CHX on the selection of staphylococci with decreased susceptibility, given the variety of products containing the compound and CHX concentrations used in clinical settings (0.5%–4%). Notably, recent research studies suggest that the decreased susceptibility of MRSA to CHX is likely to have a clinical impact.

1.4.5 Summary

MRSA is a growing threat that emphasises the need to combat resistance to biocide, especially CHX. Because it is linked to genes that resist biocide, like *qac*, this tolerance could be a link between antibiotic resistance and biocide. Lowering susceptibility to CHX requires efflux mechanisms. Co-resistance, in which genes for resistance to other antibiotics co-occur with genes for resistance to CHX, exacerbates the issue. Reports of MRSA's reduced susceptibility to CHX raise concerns, highlighting the need to investigate its possible clinical uses thoroughly. Effective public health and infection control management necessitates knowledge of and adherence to MRSA biocide tolerance.

1.5 Knowledge Gaps

In vitro studies have demonstrated the development of tolerance in bacteria exposed to sub-inhibitory concentrations of biocide components, such as triclosan, Quaternary Ammonium Compounds, and Benzalkonium chlorides. This leads to a substantial knowledge gap regarding how bacteria adapt to commercially sold disinfectants, including everyday items, such as hand sanitisers.

Even though studies on the possible dangers of biocides are growing, much remains unknown, particularly regarding the effects of sublethal biocide dosages on bacterial cells. Moreover, investigations into the intricate mechanisms underlying the development of resistance to biocides are ongoing. This implies that more extensive research is required to gain a more comprehensive understanding.

The phenomenon of post-selection persistence has received little attention because the stability of the bacterial phenotype of strains of bacteria that have evolved to increase biocide tolerance has rarely been assessed tolerance (Coombs et al., 2023). Whether the genetic changes that confer biocide tolerance continue to exist and function in the absence of biocide exposure or selective pressure remains unclear. Reports on whether genetic changes that confer biocide tolerance continue to exist and function without biocide exposure or selective pressure are still scarce. This is essential to determine whether biocide tolerance can be reversed.

1.6 Significance of Research

Bacteria becoming less sensitive to antimicrobial agents and more tolerant to biocides is a leading cause of concern. Evidence shows that bacteria may develop antibiotic resistance due to incorrect use of biocides. To guarantee that chemicals are used appropriately, it is essential to comprehend how this cross-resistance/co-resistance occurs and to evaluate the risks associated with various chemicals. Our research shows that not all biocides have the same capacity to lead to antibiotic resistance. We also elucidated the possible underlying causes of co- and cross-resistance. In addition, we performed a comprehensive assessment of the biocide risks associated with antibiotic resistance at subinhibitory concentrations

1.7 Objectives of Research

1. Characterise the Methicillin-Resistance Gene (*mecA*): Examine *mecA* gene distribution and prevalence in the *Staphylococcus aureus* strain.

2. Determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of hand sanitisers: Assess the MIC and MBC values of various hand sanitisers against parent and adapted strains. The degree of tolerance and resistance developed

by the adapted strains after the experimental evolution study can be evaluated by comparing the MIC and MBC values.

3. Study the Adaptation of *Staphylococcus aureus* to Hand Sanitisers: Analyse how *S. aureus* has evolved in response to repeated exposure to various hand sanitisers (Hexisol®, 70% Ethanol, Sepnil®). Compare the adapted strains' phenotypic changes to those of the parent strain.

4. Evaluation Patterns of Antibiotic Susceptibility: Analyse the antibiotic susceptibility profiles of both the parent and adapted strains to different antibiotic classes. Examine the development of antibiotic resistance or increased susceptibility after hand sanitiser adaptation.

5. Assessing the Stability and Growth Characteristics of Adaptive Tolerance: Assess fitness costs associated with observed antibiotic resistance and biocide tolerance. In addition, we will investigate whether the strains' adaptive tolerance to hand sanitisers remains stable after repeated subculturing in sanitiser-free media.

In this study, we postulate that co-selection for antibiotic resistance may occur because of bacterial tolerance to alcohol-based hand sanitisers at sub-inhibitory concentrations. This highlights the critical need for regulating hand sanitiser formulations and their cautious use to reduce their potential contribution to the emergence of multidrug-resistant bacterial strains.

Chapter 2

Methods & Materials

2.1 Bacterial Strain and Preparation

This investigation focused on an environmental strain of *Staphylococcus aureus* obtained from the microbial inventory of the Life Science Laboratory at BRAC University. This microbial

strain was stored in cryovials containing Tryptone Salt Agar (T1N1) and immersed in sterile paraffin oil.

The strain was revived by culturing on Tryptone Soy Agar (TSA) and incubating at 37°C. As a precaution against contamination, the strain was subcultured from a TSA plate onto a Mannitol Salt Agar (MSA) plate.

The strain was re-streaked on TSA for storage in T1N1 medium for routine use. One colony from the culture plate was isolated using an inoculating needle (sterile), stabbed to 3-5 times into T1N1 medium (contained in a sterile cryovial), and incubated at 37°C for 24 h. After incubation, 200 µL of paraffin oil was pipetted onto the top of the inoculated TIN1, secured with Parafilm, and stored at room temperature.

2.2 Media Preparation

2.2.1 Tryptone Soy Agar (TSA)

TSA (Manufacturer: Oxoid®) was utilized as a general medium for routine cell culture and bacterial enumeration. The media was prepared according to the manufacturer's instructions. TSA powder (40 g) was completely dissolved in distilled water (1 L) and heated to melt the agar before autoclaving for 15 min at 121°C. Sterilized agar was poured into Petri dishes in a laminar flow cabinet to solidify after cooling. The solidified TSA was promptly utilized or refrigerated for later use.

2.2.2 Mannitol Salt Agar (MSA)

The test organism (*Staphylococcus aureus*) was cultured on MSA for identification and as a precaution against contamination. The media was prepared according to the manufacturer's instructions. Oxoid® MSA powder (111 g) was completely dissolved in distilled water (1 L) and heated to melt the agar before autoclaving for 15 min at 121°C. Sterilized agar was poured

into Petri dishes in a laminar flow cabinet to solidify after cooling. The solidified TSA was promptly utilized or refrigerated for later use.

2.2.3 Tryptone Salt Agar (T1N1)

The sample was stored for routine use using T1N1. 20g of Agar, 10g of NaCl, and 10g of tryptone were dissolved in distilled water (1 L) and heated to a boil. 3 mL of the solution was dispensed into sterile cryovials and autoclaved at 121°C for a duration of 15min. The T1N1 was utilised immediately or refrigerated for future use.

2.2.4 Luria Bertani broth (LB)

LB was the extraction medium for bacterial DNA. The media was prepared according to the manufacturer's instructions. It was made by dissolving 25g of Hi-media® LB powder in 1 L of distilled water. The solution was then put into test tubes followed by autoclave at 121° for 15min. The test tube-containing beaker was immediately utilised or put in a refrigerator with fresh media for later use.

2.2.5 Tryptone Soya Broth (TSB)

TSB was used to measure the minimum inhibitory concentration (MIC) and perform the experimental evolution study. The media was prepared according to the manufacturer's instructions. In 1 L of distilled water, 30 g of TSB powder (manufactured by Hi-media®) was dissolved. The Duran bottle containing the broth was subjected to autoclave at 121°C for a duration of 15 min. It was then stored in the fridge or used immediately.

2.2.6 Mueller-Hinton Agar (MHA)

Antibiotic susceptibility testing was conducted using MHA. The media was prepared according to the manufacturer's instructions. MHA powder (38 g) by Hi-media® was completely dissolved in distilled water (1 L) and heated to melt the agar before autoclaving for 15 min at

121°C. Sterilized agar was poured into Petri dishes in a laminar flow cabinet to solidify after cooling. The solidified TSA was promptly utilized or refrigerated for later use.

2.2.7 Physiological Saline (0.9%)

9g of sodium chloride was dissolved in 1 L of distilled water to create the saline solution. Then, using a glass pipette, 5mL of the saline was poured into every test tube. The test tubes were autoclaved at 121°C for a duration of 15 min, then kept at room temperature or used immediately.

2.3 Source of Test Materials: Hand Sanitisers

The experiment utilised alcohol-based hand sanitisers as test materials, commonly employed for disinfection purposes in the Life Sciences laboratories of BRAC University; they were also collected from the Life Sciences laboratories of BRAC University. The list of hand sanitiser included 70% ethanol (commercial ethanol manufactured by Sigma-Aldrich), Sepnil®, and Hexisol®. Sepnil®, a product of Square Toiletries Ltd., contains 70% ethanol along with carbomer, glycerin, polyethylene glycol, TEA, water, and fragrance. According to the manufacturer (ACI Pharmaceuticals), Hexisol® is made up of 70% w/w isopropyl alcohol and 0.5% w/v chlorhexidine gluconate.

2.3.1 Preparation of Alcohol-based Hand Sanitiser Working Solution

The hand sanitisers were diluted with TSB (v/v) to achieve a range of concentrations. These concentrations were in the following ranges: 50%, 40%, 30%, 15%, 7.5%, 3.75%, 1.875%, and 0.9375%.

Concentration of hand sanitiser (v/v)	Volume of TSB (µL)	Volume of hand sanitiser (µL)
50%	5000	5000
40%	4000	6000
30%	7000	3000
15%	8500	1500
7.5%	9250	750
3.75%	9625	375
1.875%	9812	188
0.9375%	9906	94

Table 1: Composition of hand sanitiser working solution at different concentrations for 10mL

2.4 Detection of Methicillin-Resistance Gene (*mecA*)

2.4.1 Genomic DNA Extraction

Genomic DNA was isolated following the boiling method. *S. aureus* was cultured overnight in LB broth. The culture (700 µL) was placed in a microcentrifuge tube and centrifuged for 10 min at 13000 rpm. After discarding the supernatant, the cell pellet was washed using a vortex with 300 µL of distilled water. After centrifugation at 14, 000 rpm for another 5 min, the supernatant was discarded, and 200 µL of TE buffer was added. The solution was then subjected to heating at 100°C for 15 min and then cooling for 10 min. The sample was centrifuged at 14000 rpm for 5 min to remove cell debris. The supernatant was then separated and stored at -20°C for genotypic analysis.

2.4.2 Polymerase Chain Reaction (PCR) for Detecting Methicillin-Resistance Gene (*mecA)*

The method for detecting the *mecA* gene was executed by the protocol outlined by Igbinosa et al.(2016). The PCR reaction mixture had a volume of 13 µL and consisted of 2 µL of genomic DNA, 6 µL of EmeraldAmp® GT PCR Master Mix, 3 µL of nuclease-free water, and 1 µL each of the *mecA* primers. The primers used for the *mecA*-F and *mecA*-R genes were as follows: *mecA*-F primer sequence was 5'-TCCAGATTACAACTTCACCAGG-3', while the *mecA*-R primer sequence was 5'-CCACTTCATATCTTGTAACG-3'. The resulting amplicon size was 162 base pairs. The amplification procedure was carried out in 40 cycles, with these stages: initial denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min, followed by cooling at 4 °C.

2.4.3 Gel Electrophoresis

The amplicon was visualised using electrophoresis on a 1% agarose gel with the addition of ethidium bromide in a concentration of 0.5 mg/L. The electrophoresis process was carried out at a voltage of 100 V for 60 min (Igbinosa et al., 2016).The gel run was performed in a 0.5x TAE buffer (1 mM EDTA, 20 mM Na-acetate, and 40 mM Tris-HCl) with a pH of 8.5. The resulting gel was visualised using a UV transilluminator. The experiment utilised a 1Kb DNA ladder (Bio-Helix Co., Ltd.), a positive control (verified MRSA strain), and a negative control of E. coli O157:H7.

2.5 Experimental Evolution study (Adaptation to Hand Sanitisers)

This study followed the method described in a previous study (Gadea et al., 2017). A range of Hexisol®, Sepnil®, and 70% ethanol concentrations were prepared (see section 2.3). An aliquot of 100 µL from an overnight bacterial culture was added to 10 mL of TSB supplemented with hand sanitiser. The inoculums were incubated at 37°C for 48 h. A subculture (1000 µL)

from the tube with the highest concentration of hand sanitiser that produced turbidity after incubation was transferred into a new tube with the same concentration of hand sanitiser and a higher concentration (an increase of 0.125 for Sepnil® and 70% ethanol, and a 2-fold increase for Hexisol®). The subcultures were incubated at 37°C for 48 h. After the incubation, cultures grown in the higher concentration of hand sanitiser were chosen to repeat the above protocol. The process was repeated, transferring subcultures from the previous tube with documented growth into tubes with the same concentration and the next highest concentration of hand sanitiser, until no visible growth was observed after 72 h of incubation (for death confirmation). The control strain was grown in a hand-sanitiser-free medium. A suspension from the final tube containing growth was obtained and spread on a TSA plate for further analysis (for each evolved strain). Bacterial growth was also collected and suspended in 1 mL of TSB with 20% glycerol and preserved at -20°C. The evolved and control strains were subjected to Antibiotic Susceptibility Test (AST), Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and growth curve assays to observe phenotypic changes.

The stability of adaptive tolerance was assessed by subjecting the culture to repeated culturing in hand sanitiser-free TSB every 24 h for 10 days. Subsequently, AST (if the evolved strain had acquired co-selection to any antibiotics), MIC (if evolved strain had acquired increased tolerance to hand sanitisers), and MBC tests were performed on the evolved strains.

2.6 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Alcohol-based Hand Sanitisers

The MIC was determined for the parental strain and the three evolved strains. Although there is no CLSI technique for assessing hand sanitisers, this test was conducted using the macrodilution broth dilution method described by CLSI for antimicrobial agents (Clinical and Laboratory Standards Institute, 2022), with a full inhibitory endpoint at 18–20 h of incubation.

A series of dilutions was performed using TSB to prepare various concentrations of Sepnil®, 70% ethanol, and Hexisol® (see section 2.3). These concentrations ranged from 50%, 40%, 30%, 15%, 7.5%, 3.75%, 1.875%, and 0.9375% (v/v).

Direct broth or saline suspensions were prepared using colonies taken from an 18- to 24-hour culture. The suspension was subsequently adjusted until it reached turbidity levels consistent with the 0.5 McFarland standard. The adjusted suspension was diluted in broth within 15 min of production, so every tube contained approximately 5×10^5 CFU/mL. This was accomplished by diluting the suspension (equivalent to 0.5 McFarland) to a ratio of 1:150, yielding a tube with approximately 1×10^6 CFU/mL. A 1:1 volume mixture of adjusted inoculum and diluted hand sanitiser was prepared after standardisation within a 15 min window. This led to a 1:2 dilution of the inoculum and each hand sanitiser concentration. These tubes were incubated at 35°C for a duration of 20 h. A positive control comprising the inoculum and broth, and negative controls containing only TSB supplemented with hand sanitiser were also used.

The MIC, the lowest amount of antimicrobial agent required to halt organism growth in test tubes, was determined through visual examination. Growth endpoints were identified by comparison with positive growth-control tubes. Proper growth in the control tube was essential for validating the test. It was established that the MIC was the concentration of hand sanitiser needed to halt visible growth effectively.

Visually determining the MIC became difficult owing to the particular properties of the hand sanitisers Hexisol® and Sepnil®. The MBC values were obtained to verify the MIC values for these products. The MBC, which represents the lowest concentration of hand sanitiser required to remove 99.9% of the initial bacterial population, is determined by plating and enumerating the concentrations equal to or greater than the MIC (in this experiment, all the concentrations were plated).

Following the determination of MIC values, 100 µL of the contents from the tubes was spread onto hand-sanitiser-free TSA plates using the spread-plating technique. The plates were kept for incubation for 24 h at 35°C. The lowest concentration of hand sanitiser at which bacterial growth was absent on TSA plates was defined as the MBC. The maximum hand sanitiser concentration at which bacterial growth was still present on the TSA plate, just before reaching the MBC, was determined to be the MIC.

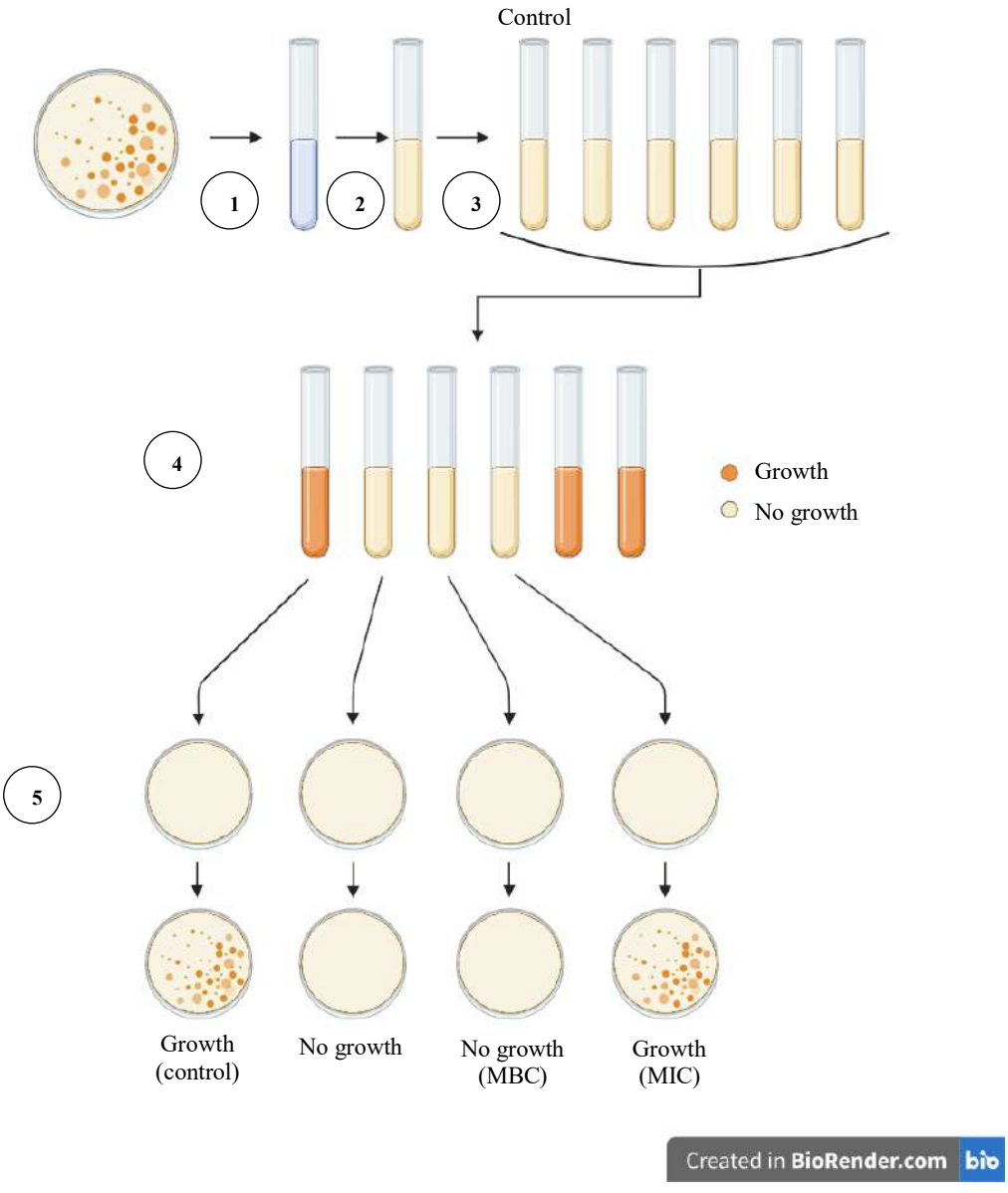


Figure 3: Confirming MIC values from MBC. 1: The direct colony suspension method was used to prepare inoculum in physiological saline with turbidity equal to 0.5 McFarland standard. 2: Dilute inoculum by 1:150 using TSB. 3: Different hand sanitiser concentrations were prepared by diluting with TSB and mixing the adjusted inoculum and hand sanitiser containing TSB in 1:1. 4: The test tubes were incubated at 35°C for 16 to 20 h. 5: All the tubes were spread in biocide-free TSA. Created with BioRender.com.

2.7 Antibiotic Susceptibility Test

The MICs of a broad range of antibiotic classes were determined to determine the presence of co-selection. The antibiotic resistance pattern was assessed via AST for the parent, and three evolved strains. The standard disc diffusion method, also known as the Kirby-Bauer test, was used to assess the resistance or sensitivity of the sample to various antibiotics. On TSA agar, bacteria were grown for 18 to 24 hours before being suspended in sterilised physiological saline solution, and then turbidity was calibrated to a 0.5 McFarland standard (~10^8 cfu/mL). A sterile cotton swab was used to lawn the culture suspension onto MHA plates, and then the antibiotics were placed on the plate using a tweezer. The following were the antibiotic classes, antimicrobial agent types, and disk contents: Aminoglycosides (Amikacin 30 µg, Gentamycin 10 µg); Carbapenem (Meropenem 10 µg); Cephalosporin (Ceftazidime 30 µg); Fluoroquinolone (Ciprofloxacin 5 µg); Lincosamides (Clindamycin 30 µg); Oxazolidinone (Linezolid 30 µg); Macrolides (Azithromycin 30 µg, Erythromycin 15 µg); β-lactam (Methicillin 5 µg); Phenicols (Chloramphenicol 30 µg); and Tetracycline (Tetracycline 30 µg). The observed results were obtained over a 24-hour incubation period at a temperature of 37°C. The diameter of the inhibition zone surrounding each disc was measured (using a scale) according to the protocol by the Clinical Laboratory Standards Institute, and it was classified as Resistant (R), Intermediate (I), or Sensitive (S) (Hudzicki, 2012). The mean value of three

separate experiments was determined, the standard deviation of the mean was calculated, and a bar graph was plotted (for each hand sanitiser) using Microsoft Excel version 16.77.

2.8 Growth Curve Assay

To evaluate the in vitro fitness of the evolved strains and compare them to the parent strain, growth curve assays were conducted. Diluting overnight cultures of the strains with TSB yielded a final inoculum of 1% culture (OD600: ~0.01). The optical densities at 600 nm (OD600) were measured using the Thermo Scientific™ BioMate™ 3S UV-Visible spectrophotometer after 2, 4, 6, and 24 h incubation at 37 °C. Average absorbance values were analysed from triplicate experiments.

2.9 Statistical Analysis

Bar plots were used to visualise the data from the AST results for both the parent and evolved strains, with error bars reflecting the standard deviation (SD). The error bars for both were evaluated to ascertain a statistically significant difference between the parent strain and hand sanitiser-adapted strains. Fewer overlapping error bars indicate a lower likelihood of significance, whereas non-overlapping bars indicate potential significance, although certainty is not assured. Overlapping error bars indicate non-significant differences. A paired t-test was then performed to draw a conclusion with a *p*-value of <0.05, which was considered as statistically significant.

Using the Statistical Package for Social Sciences (SPSS) software (IBM Corporation, USA), a paired t-test was performed to evaluate any statistically significant differences in growth capacity between the parent and evolved strains. The null hypothesis that growth capacity was not different between the parent and evolved strains was rejected at a p-value <0.05.

Chapter 3

Results

3.1 Polymerase Chain Reaction (PCR) for Detecting Methicillin-Resistance Gene (*mecA)*

The PCR amplification and subsequent gel electrophoresis revealed the presence of the *mecA* gene in the *Staphylococcus aureus* strain used in this experiment (Figure 4). The amplicon comprised 162 base pairs and was found at the expected position.

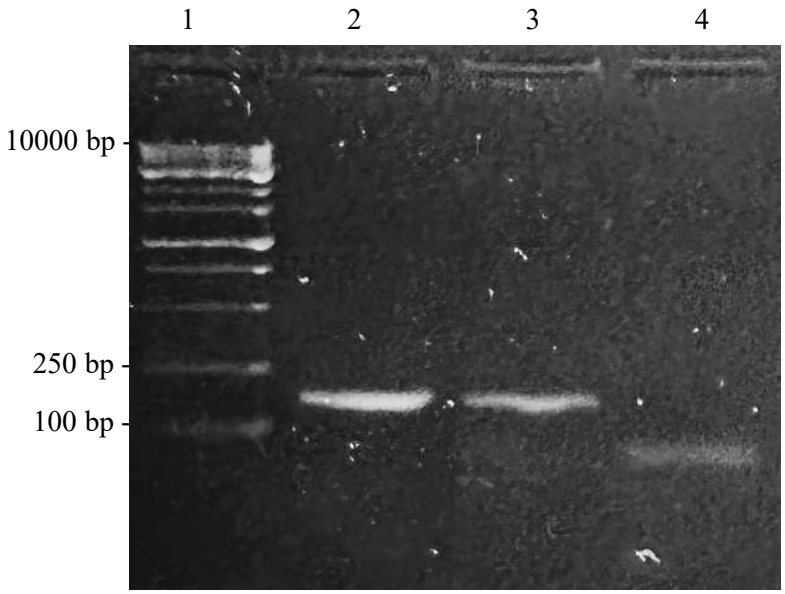


Figure 4: Gel electrophoresis (1% agarose) of PCR amplicon after amplification of the *mecA* gene. Lane 1: 1Kb DNA ladder. Lane 2: Positive control (verified MRSA strain). Lane 3: Test sample (*Staphylococcus aureus*). Lane 4: Negative control (*E. coli O157:H7*).

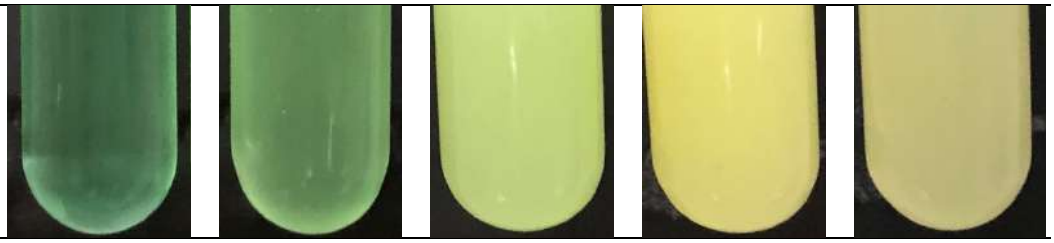
3.2 Hexisol®

The adaptation period of the test sample *Staphylococcus aureus* to Hexisol® lasted 12 days.

3.2.1 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

In comparison to the parent strain, the MIC of Hexisol® against the evolved strain exhibited a 4 -fold increase, whereas the MIC remained the same as that of the control strain. Moreover, the increased adaptive tolerance to Hexisol® remained stable in the evolved strain after 10 subcultures in an Hexisol®-free broth. The experiment had three repetitions, each of which yielded consistent results.

Table 2: MIC of Hexisol® against the parent strain.

Concentration	50%	40%	30%	15%	7.5%
Results					
Concentration	3.75%	1.875%	0.9375%	Positive control	Negative control
Results					

MIC could not be visually determined due to the nature of Hexisol® (turbidity was observed in the negative control). Hence, MBC was done using these results to confirm the MIC (Figure 5).

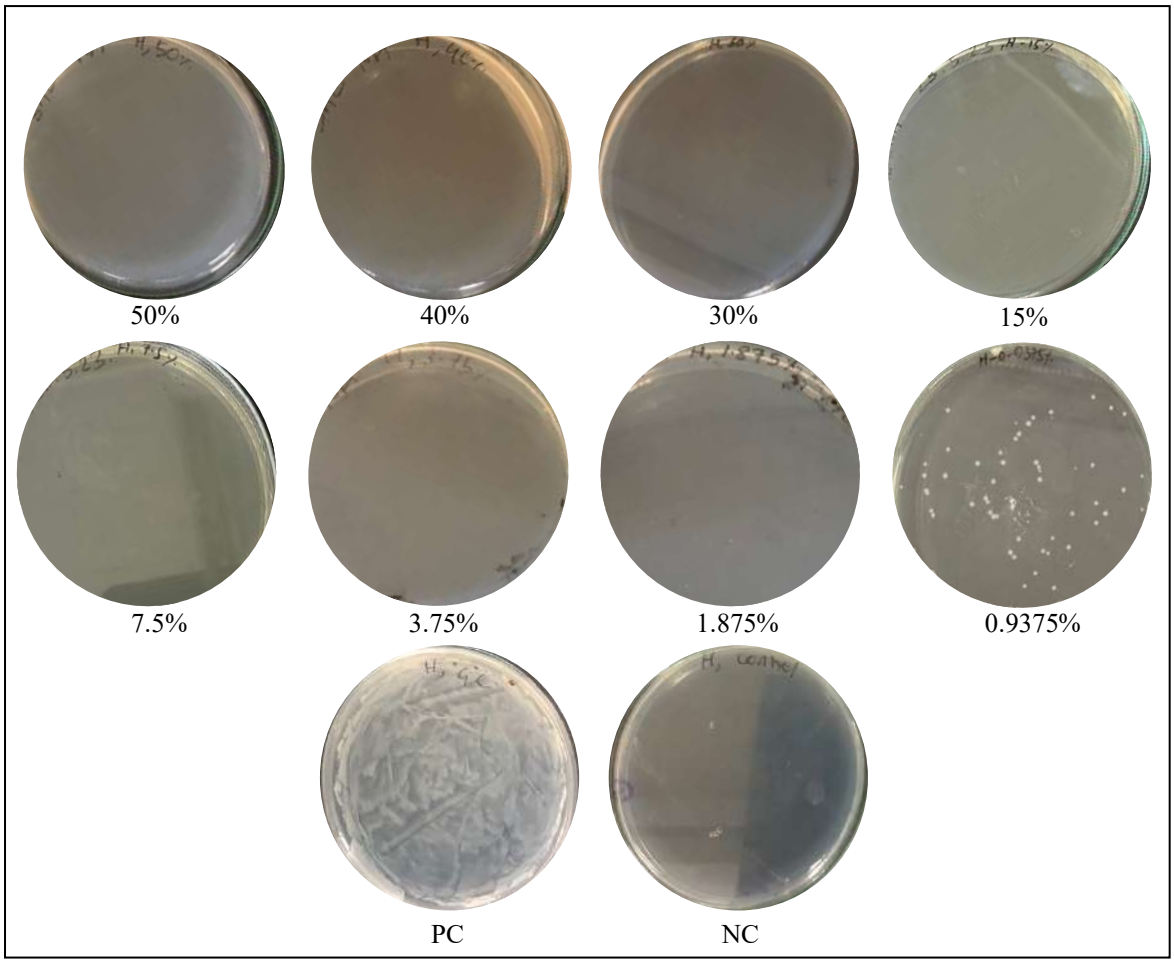
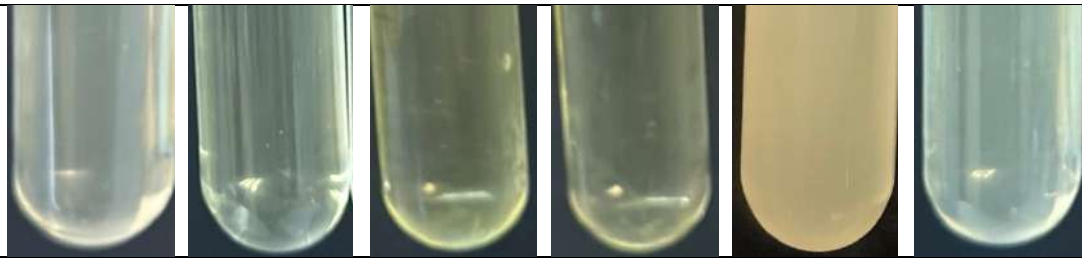


Figure 5: MBC results of Hexisol® against the parent strain.

Based on Figure 5, the MBC of Hexisol® against the parent strain was determined to be 1.875% (using the procedure shown in Figure 3) as this was the lowest concentration of Hexisol® that showed no bacterial growth on its plate. The highest concentration of Hexisol® (before reaching the MBC) at which bacterial growth was still present on the plate was 0.9375% (v/v). Thus, the MIC of Hexisol® was confirmed to be 0.9375%.

Table 3: MIC of Hexisol® against the Hexisol®-adapted strain.

Concentration	7.5%	3.75 %	1.875 %	0.9375%	Positive control	Negative control
Results						

MIC could not be visually determined due to the nature of Hexisol® (turbidity was observed in the negative control). Hence, MBC was done using these results to confirm the MIC (Figure 6).

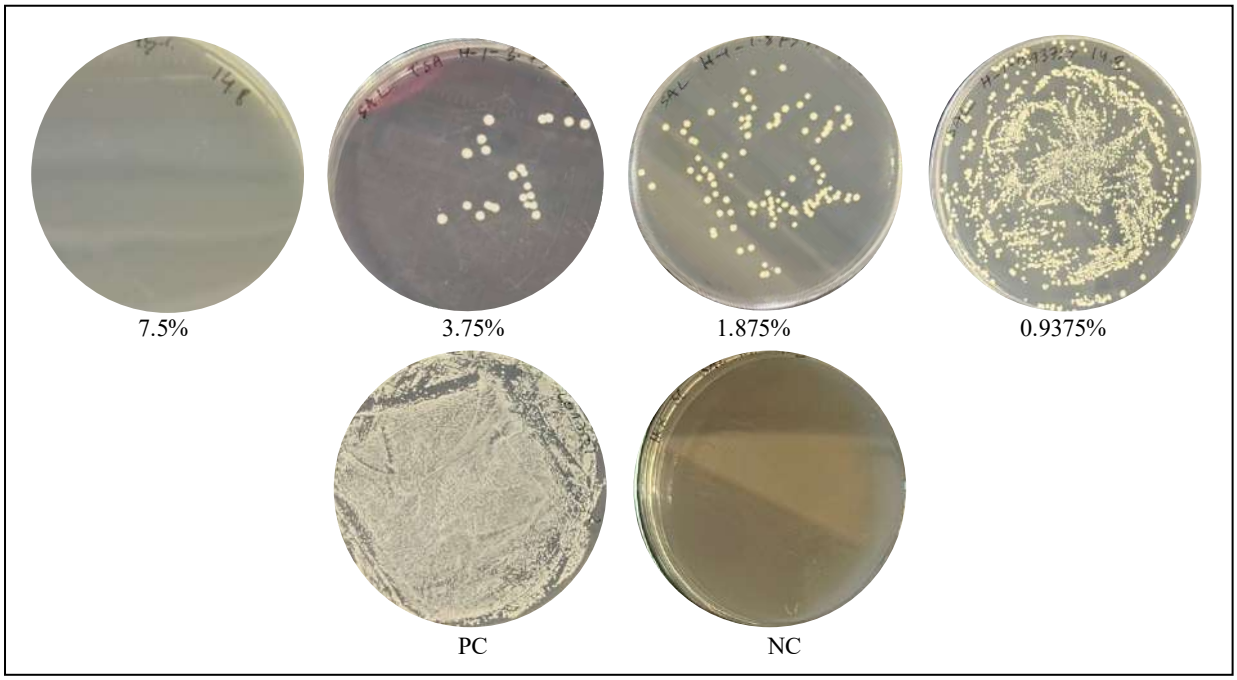
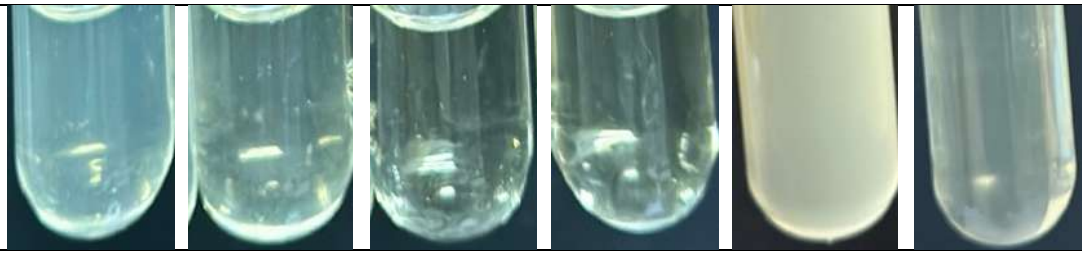


Figure 6: MBC results of Hexisol® against the Hexisol®-adapted strain.

The MBC of Hexisol® against the Hexisol®-adapted strain was determined to be 7.5% as no bacterial growth was observed on the corresponding plate (Figure 6). The highest concentration of Hexisol® at which bacterial growth was still visible (before reaching MBC) was on the plate

with 3.75% Hexisol® (v/v), confirming the MIC of Hexisol® as 3.75%. Thus, the MIC of Hexisol® increased by 4-fold in the evolved strain.

Table 4: MIC of Hexisol® against the Hexisol®-adapted strain after 10 d of subculture in Hexisol®-free broth.

Concentration	7.5%	3.75%	1.875%	0.9375%	Positive control	Negative Control
Results						

MIC could not be visually determined due to the nature of Hexisol® (turbidity was observed in the negative control). Hence, MBC was done using these results to confirm the MIC (Figure 7).

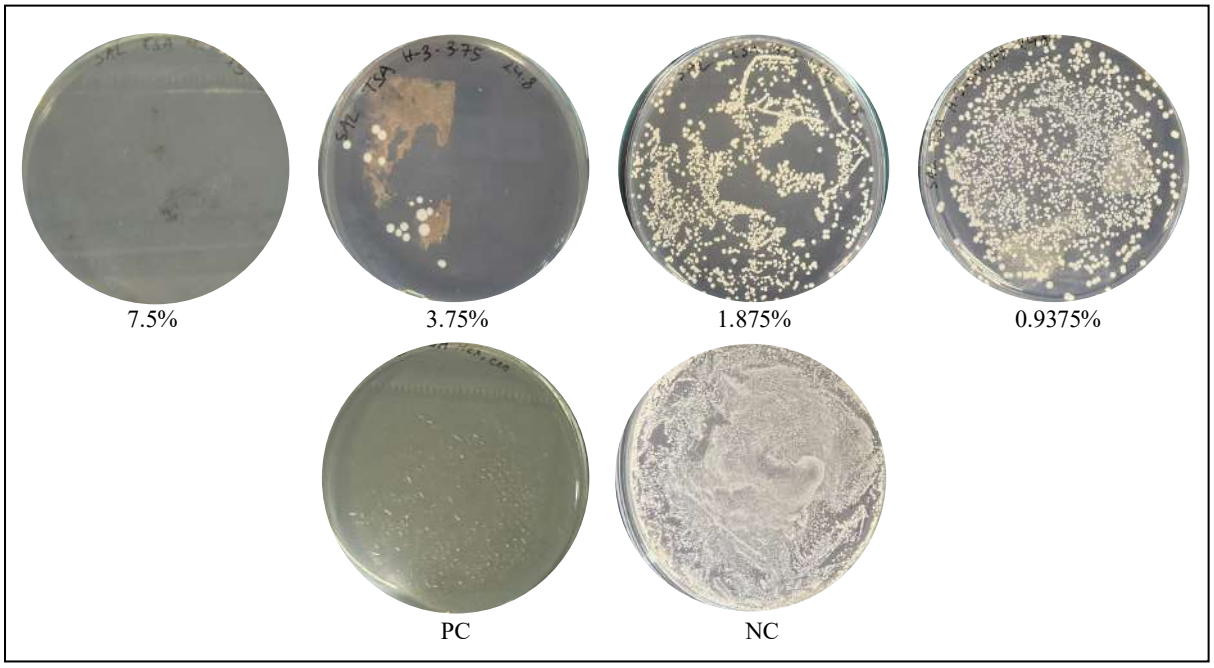


Figure 7: MBC results of Hexisol® against the Hexisol®-adapted strain after 10 days of subculture in Hexisol®-free broth.

As shown in Figure 7, it was determined that the MBC of Hexisol® against the Hexisol®-adapted strain after 10 d of repeated subculture in Hexisol®-free broth was 7.5% as no bacterial growth was observed on the plate. Thus, the MIC of Hexisol® was confirmed to be 3.75% (the highest concentration of Hexisol® showing bacterial growth), and the increased tolerance of the Hexisol®-adapted strain against Hexisol® remained stable after 10 d.

3.2.2 Antibiotic Susceptibility Test

Table 5: Resistance pattern of *S. aureus* before and after the adaptation to Hexisol®.

Antibiotic	Zone of inhibition (mm)	
	Parent Strain	Hexisol®-adapted Strain
MRP	38 (S)	38 (S)
AZM	32 (S)	0 (R)
TE	31 (S)	31 (S)
MET	22 (S)	10 (R)
GEN	29 (S)	36 (S)
C	28 (S)	30 (S)
CIP	31 (S)	37 (S)
CD	31 (S)	30 (S)
AK	29 (S)	37 (S)
LZ	32 (S)	37 (S)
CAZ	0 (R)	0 (R)
E	32 (S)	0 (R)

S: susceptible; R: resistant; MRP: meropenem; AZM: azithromycin; TE: tetracycline; MET: methicillin; GEN: gentamycin; C: chloramphenicol; CIP: ciprofloxacin; CD: clindamycin; AK: amikacin; LZ: linezolid; CAZ: Ceftazidime; E: Erythromycin.

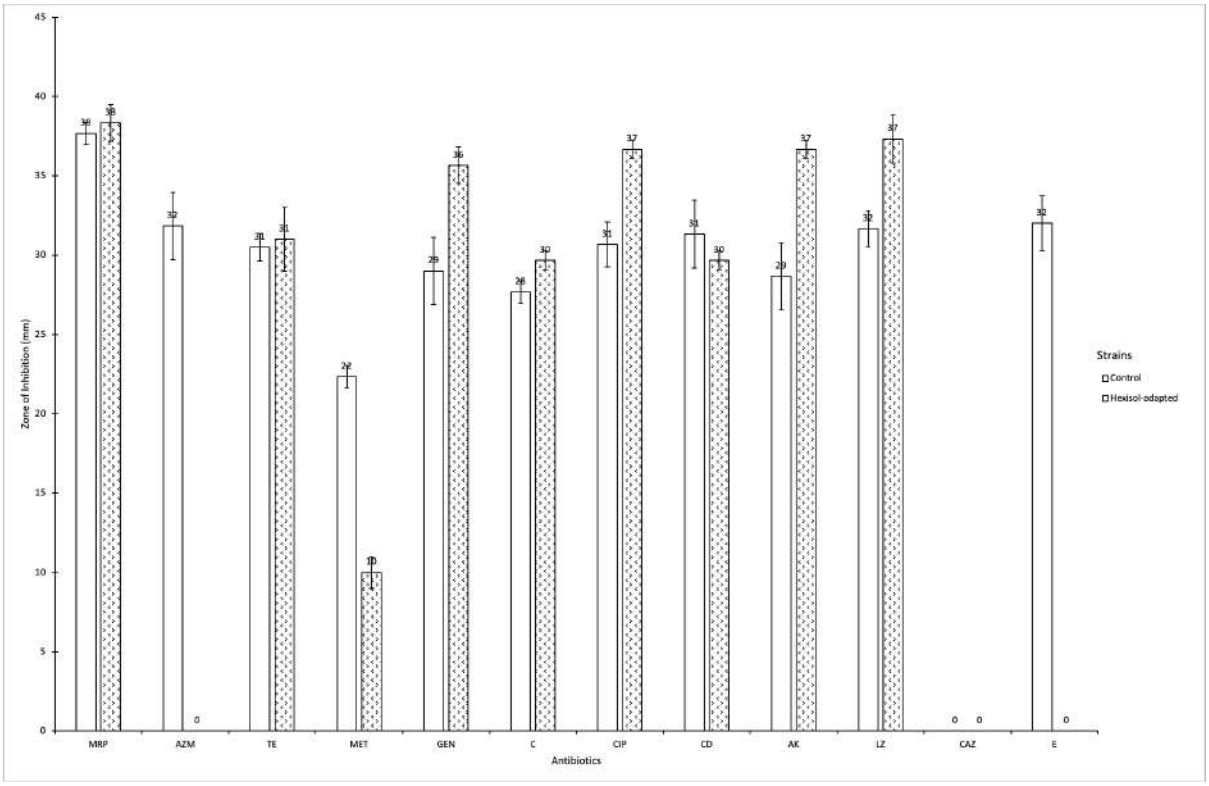


Figure 8: Comparative analysis of the zone of inhibition (mm) measured for the parent strain and Hexisol®-adapted strains against antibiotics. MRP: meropenem; AZM: azithromycin; TE: tetracycline; MET: methicillin; GEN: gentamycin; C: chloramphenicol; CIP: ciprofloxacin; CD: clindamycin; AK: amikacin; LZ: linezolid; CAZ: Ceftazidime; E: Erythromycin.

The data (Table 5, Figure 8) represent the mean zone diameter values obtained from three independent experiments, and the standard deviation of the mean is shown by the vertical error bar. The AST results for the control strain were consistent with those for the parent strain. The Hexisol®-adapted strain showed a significant increase in resistance to azithromycin, methicillin, and erythromycin. The zone of inhibition of AZM and E reached zero (indicating complete resistance), and a statistically significant increase was observed in resistance to MET compared to the parent strain ($p<0.05$). In contrast, the Hexisol®-adapted strain remained susceptible to most antibiotics, except for ceftazidime, to which it remained resistant. There was an increase in susceptibility to most antibiotics in the Hexisol®-adapted strain compared to the parent strain; however, the difference was statistically significant ($p<0.05$) for only four

antibiotics: gentamycin, ciprofloxacin, amikacin, and linezolid. The highest increase in susceptibility was observed for linezolid.

Table 6: Resistance patterns of the parent, Hexisol®-adapted, and Hexisol®-adapted strains after being subjected to 10 d of subculture in Hexisol®-free broth.

Antibiotic	Zone of inhibition (mm)		
	Parent Strain	Hexisol®-adapted Strain	Hexisol®-adapted Strain (after 10d)
AZM	32 (S)	0 (R)	32 (S)
MET	22 (S)	10 (R)	24 (S)
E	32 (S)	0 (R)	31 (S)

S, susceptible; R, resistant; AZM: azithromycin: MET, methicillin; E: Erythromycin.

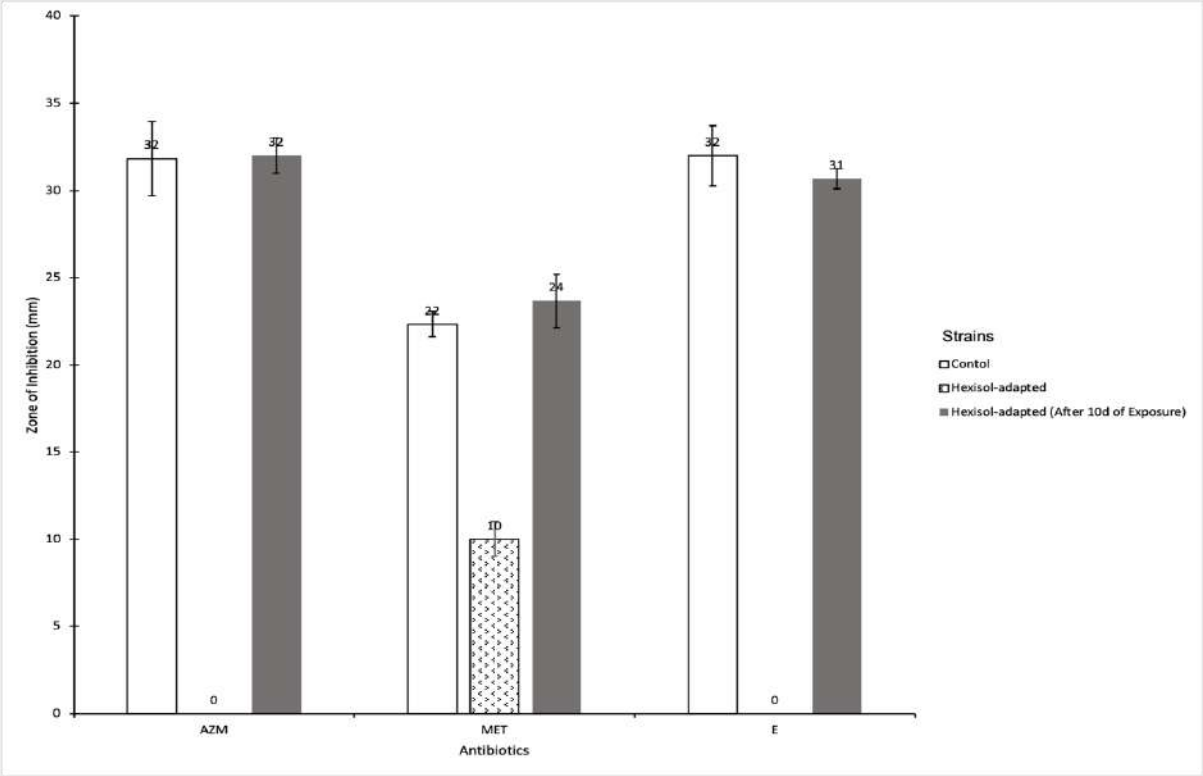


Figure 9: Comparative analysis of the zone of inhibition (mm) measured for the parent, Hexisol®-adapted, and Hexisol®-adapted strains after being subjected to 10 d of subculture in

Hexisol®-free broth against antibiotics. AZM: azithromycin: MET, methicillin; E: Erythromycin.

The previously observed adaptive cross-resistance of the Hexisol®-adapted strain against azithromycin, methicillin, and erythromycin did not remain stable after 10 d of repeated subculturing in Hexisol®-free broth. The zone of inhibition measured for the Hexisol®-adapted strain against these 3 antibiotics showed an increase after 10 d of subculture, as depicted in Figure 9, and was similar to the values observed for the parent strain; no statistically significant differences between these measured values were found.

3.2.3 Growth Curve Assay

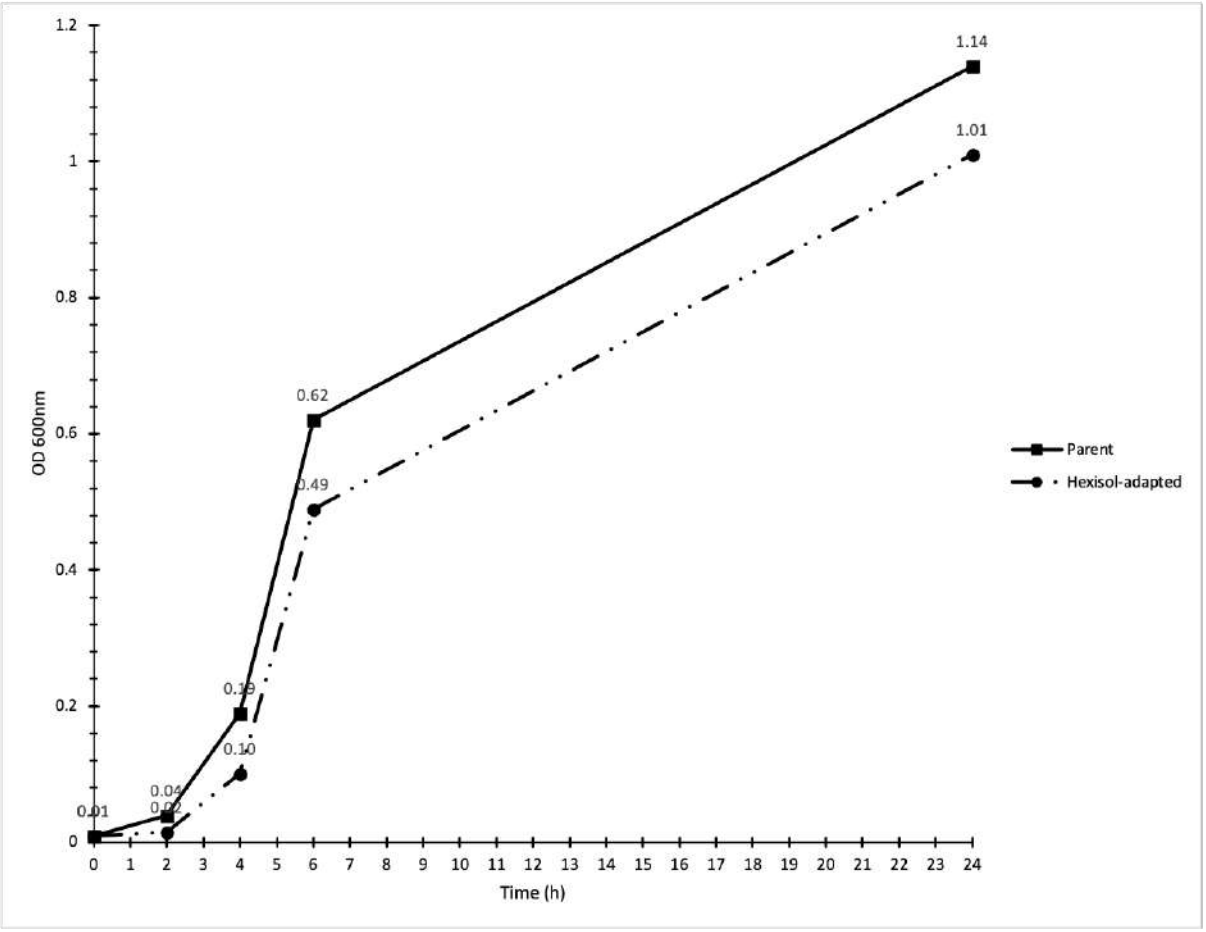


Figure 10: Growth capacity of parent strain and Hexisol®-adapted strain.

A similar growth trend was noted between the parent and Hexisol®-adapted strains (Figure 10). Based on Figure 10, the Hexisol®-adapted strain ($M = 0.4, SD = 0.48$) exhibited a statistically significant decrease in bacterial growth rate (lower optical density) in comparison to the parent strain ($M = 0.32, SD = 0.43$), $t(4) = 2.77$, $p<0.05$.

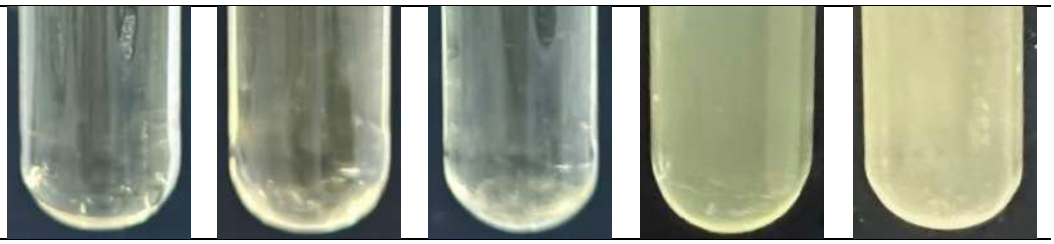
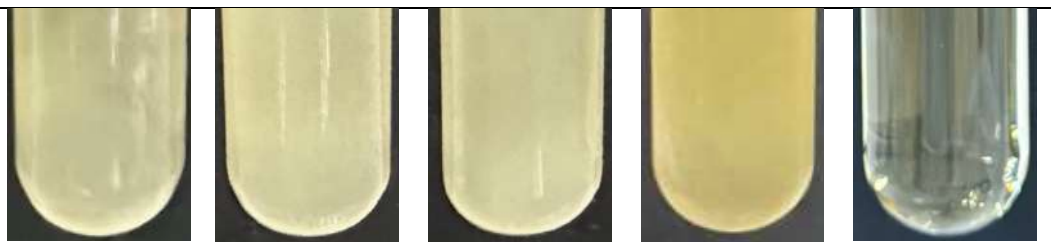
3.3 70% Ethanol

The adaptation period of the test sample *S. aureus* to 70% ethanol lasted 12 days.

3.3.1 Determination of Minimum Inhibitory Concentration (MIC)

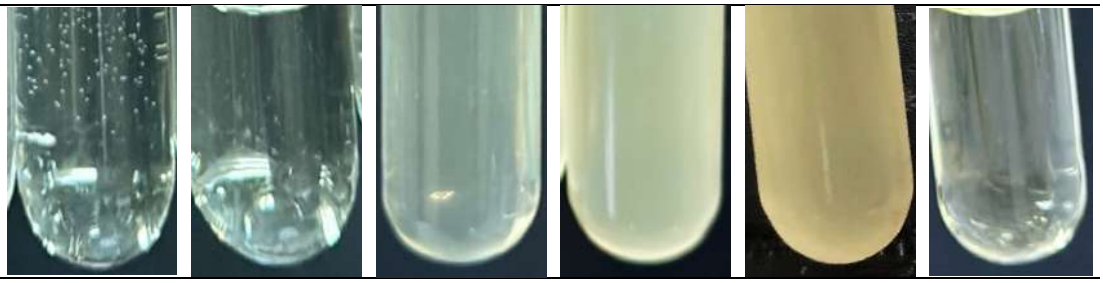
The MIC of 70% ethanol could be visually determined so performing MBC was not needed. Compared to the parent strain, the MIC of 70% ethanol against the evolved strain exhibited a 1.33-fold increase, whereas the MIC remained the same as that of the control strain. Moreover, the increased adaptive tolerance to 70% ethanol remained stable in the evolved strain after 10 subcultures in an ethanol-free broth. The experiment had three repetitions, each of which yielded consistent results.

Table 7: MIC results of 70% ethanol against the parent strain.

Concentration	50%	40%	30%	15%	7.5%
Results					
Concentration	3.75%	1.875%	0.9375%	Positive control	Negative control
Results					

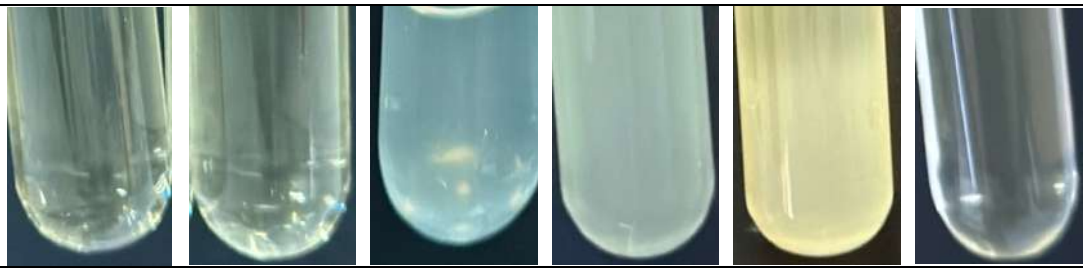
As shown in Table 7, the 30% concentration (v/v) of 70% ethanol did not result in turbidity. Thus, the initial MIC value of 70% ethanol on parent strain is 30% (v/v).

Table 8: MIC results of 70% ethanol against the ethanol-adapted strain.

Concentration	50%	40%	30%	15%	Positive control	Negative Control
Results						

As shown in Table 8, the 40% concentration (v/v) of 70% ethanol did not result in turbidity. Thus, the MIC of 70% ethanol against S. aureus increased 1.33-fold in the evolved strain.

Table 9: MIC results of 70% ethanol against the ethanol-adapted strain after 10 d of subculture in ethanol-free broth.

Concentration	50%	40%	30%	15%	Positive control	Negative Control
Results						

As shown in Table _, the 40% concentration (v/v) of 70% ethanol did not result in turbidity. Thus, the increased adaptive tolerance to 70% ethanol remained stable after inoculation in ethanol-free broth for 10 subcultures.

3.3.2 Antibiotic Susceptibility Test

Table 10: Resistance pattern of *S. aureus* before and after the adaptation to 70% ethanol.

Antibiotic	Zone of inhibition (mm)	
	Parent Strain	Ethanol-adapted Strain
MRP	38 (S)	41 (S)
AZM	32 (S)	35 (S)
TE	31 (S)	35 (S)
MET	22 (S)	26 (S)
GEN	29 (S)	31 (S)
C	28 (S)	33 (S)
CIP	31 (S)	36 (S)
CD	31 (S)	39 (S)
AK	29 (S)	31 (S)
LZ	32 (S)	32 (S)
CAZ	0 (R)	0 (R)
E	32 (S)	32 (S)

S: susceptible; R: resistant; MRP: meropenem; AZM: azithromycin; TE: tetracycline; MET: methicillin; GEN: gentamycin; C: chloramphenicol; CIP: ciprofloxacin; CD: clindamycin; AK: amikacin; LZ: linezolid; CAZ: Ceftazidime; E: Erythromycin.

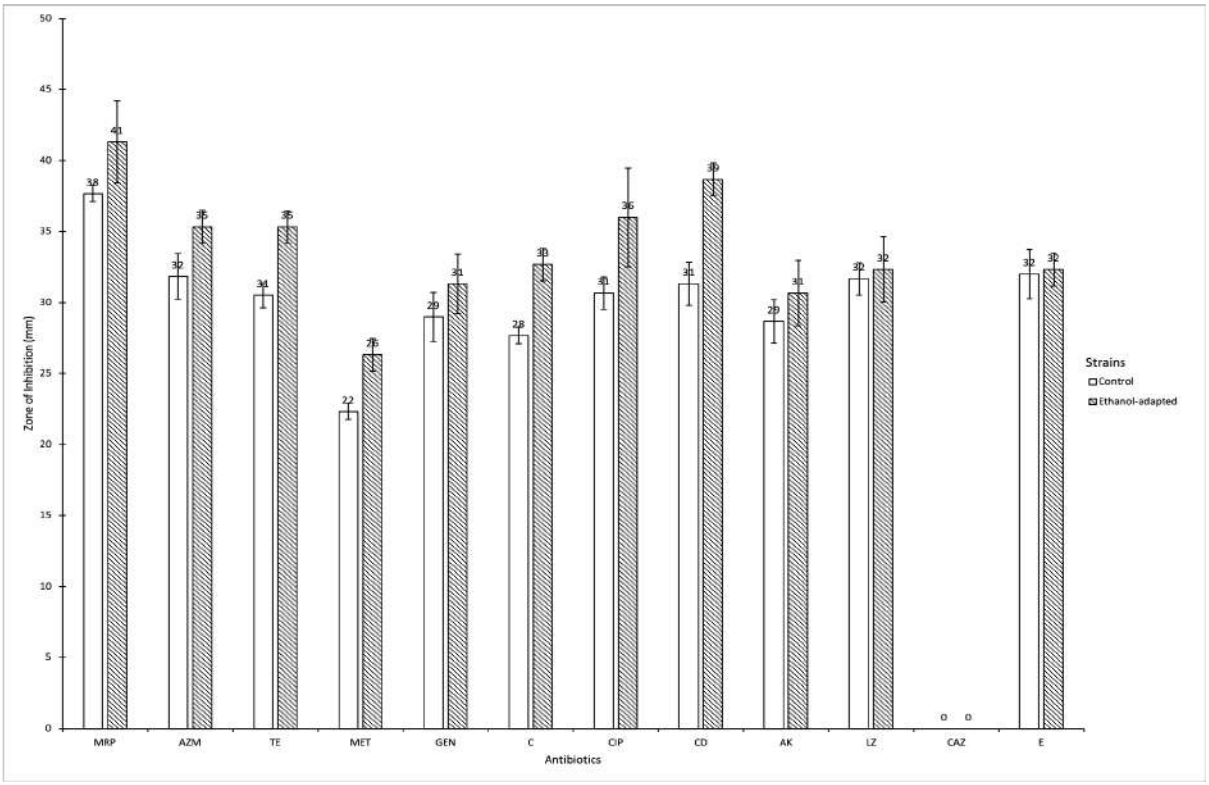


Figure 11: Comparative analysis of the zone of inhibition (mm) measured for the parent strain and ethanol-adapted strains against antibiotics. MRP: meropenem; AZM: azithromycin; TE: tetracycline; MET: methicillin; GEN: gentamycin; C: chloramphenicol; CIP: ciprofloxacin; CD: clindamycin; AK: amikacin; LZ: linezolid; CAZ: Ceftazidime; E: Erythromycin.

The data (Table 10, Figure 11) represent the mean zone diameter values obtained from three independent experiments, and the standard deviation of the mean is shown by the vertical error bar. The AST results for the control strain were consistent with those for the parent strain. The ethanol-adapted strain remained susceptible to all antibiotics except ceftazidime, to which it remained resistant. There was an increase in susceptibility to most antibiotics (except ceftazidime) in the ethanol-adapted strain compared to that in the parent strain; however, the difference was statistically significant (p<0.05) for only four antibiotics: tetracycline, methicillin, chloramphenicol, and clindamycin. The highest increase in susceptibility was observed for clindamycin.

3.3.3 Growth Curve Assay

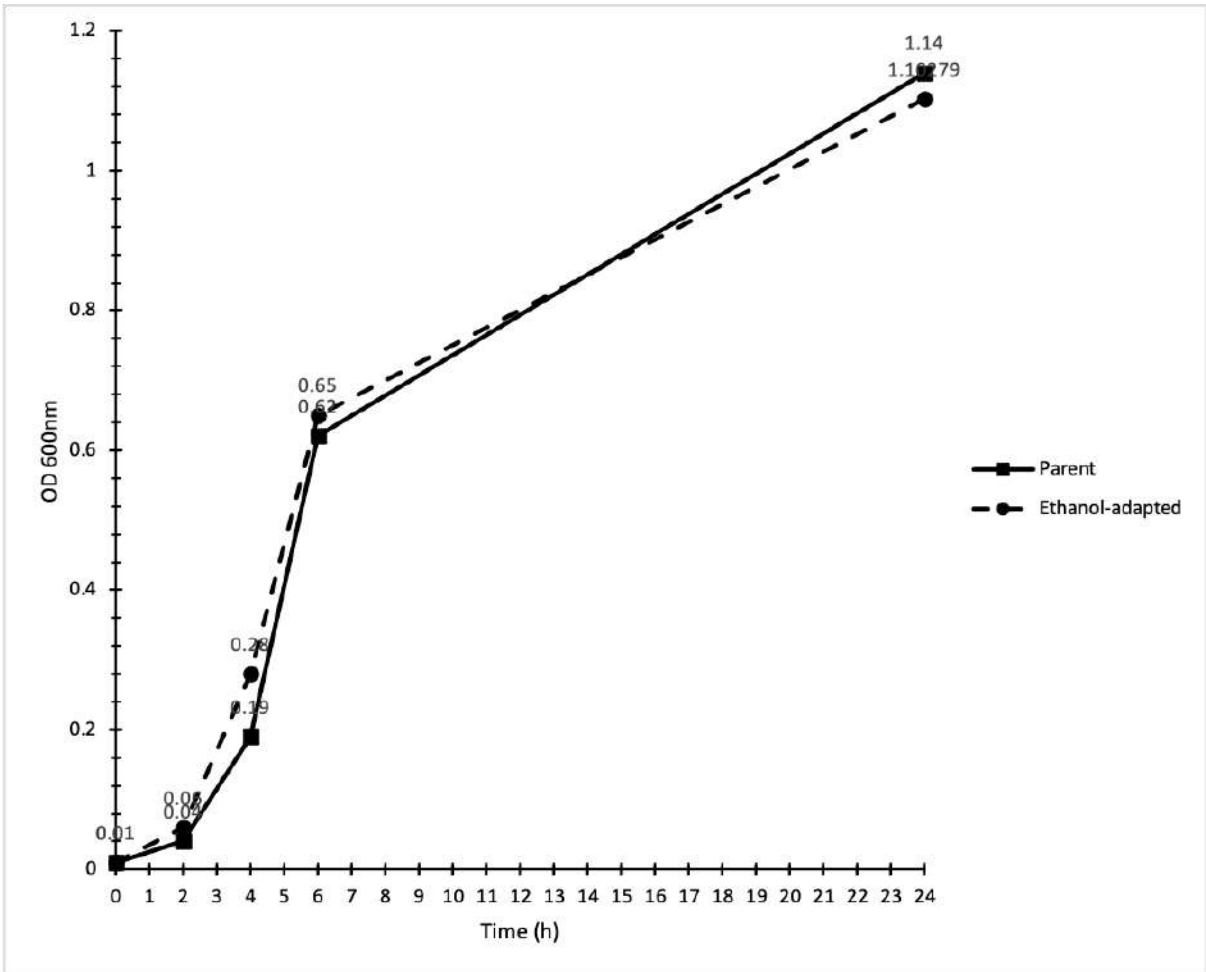


Figure 12: Growth capacity of parent strain versus Ethanol-adapted strain.

A similar growth trend was noted between the parent and ethanol-adapted strains (Figure 12). The ethanol-adapted strain showed slightly higher optical density values (and thus, growth rate) within the initial 6 h. However, there was no statistically significant differences in growth capacity of the parent ($M = 0.4$, $SD = 0.48$) and ethanol-adapted strains ($M = 0.37$, $SD = 0.44$), $t(4) = 1.49$, $p = 0.211$.

3.4 Sepnil®

The adaptation period of the test sample *S. aureus* to Sepnil® lasted 12 days.

3.4.1 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Compared to the parent strain, the MIC of Sepnil® against the evolved strain exhibited a 1.25-fold increase, whereas the MIC remained the same as that of the control strain. Moreover, the increased adaptive tolerance to Sepnil® remained stable in the evolved strain after 10 subcultures in an Sepnil®-free broth. The experiment had three repetitions, each of which yielded consistent results.

Table 11: MIC results of Sepnil® against the parent strain.

Concentration	50%	40%	30%	15%	7.5%
Results					
Concentration	3.75%	1.875%	0.9375%	Positive control	Negative control
Results					

MIC could not be visually determined due to the nature of Sepnil® (turbidity was observed in the negative control). Hence, MBC was done using these results to confirm the MIC (Figure 13).

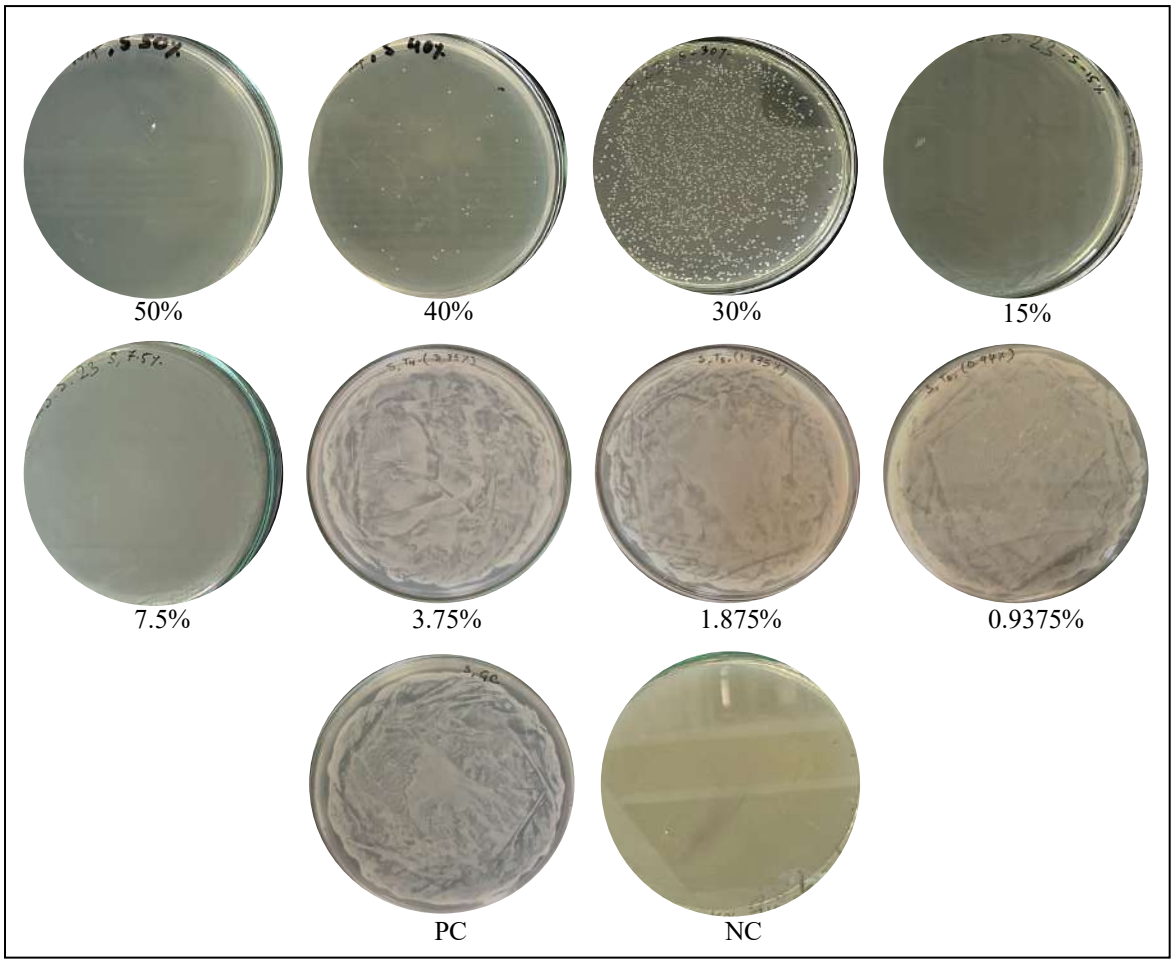
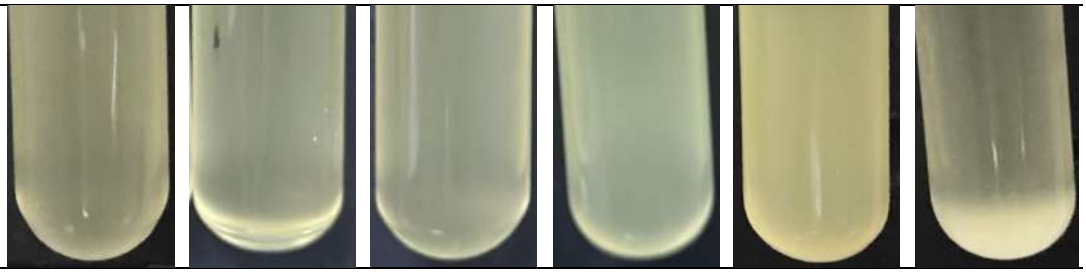


Figure 13: MBC results of Sepnil® against the parent strain.

As shown in Figure13, MBC of Sepnil® against the parent strain was determined to be 50% (using the procedure shown in Figure 3) as this was the lowest concentration of Sepnil® that showed no bacterial growth on its plate. The highest concentration of Sepnil® (before reaching the MBC) at which bacterial growth was still present on the plate was 40% (v/v). Thus, the MIC of Sepnil® was confirmed to be 40%.

Table 12: MIC of Sepnil® against the Sepnil®-adapted strain.

Concentration	60%	50%	40%	30%	Positive control	Negative Control
Results						

MIC could not be visually determined due to the nature of Sepnil® (turbidity was observed in the negative control). Hence, MBC was done using these results to confirm the MIC (Figure 14).

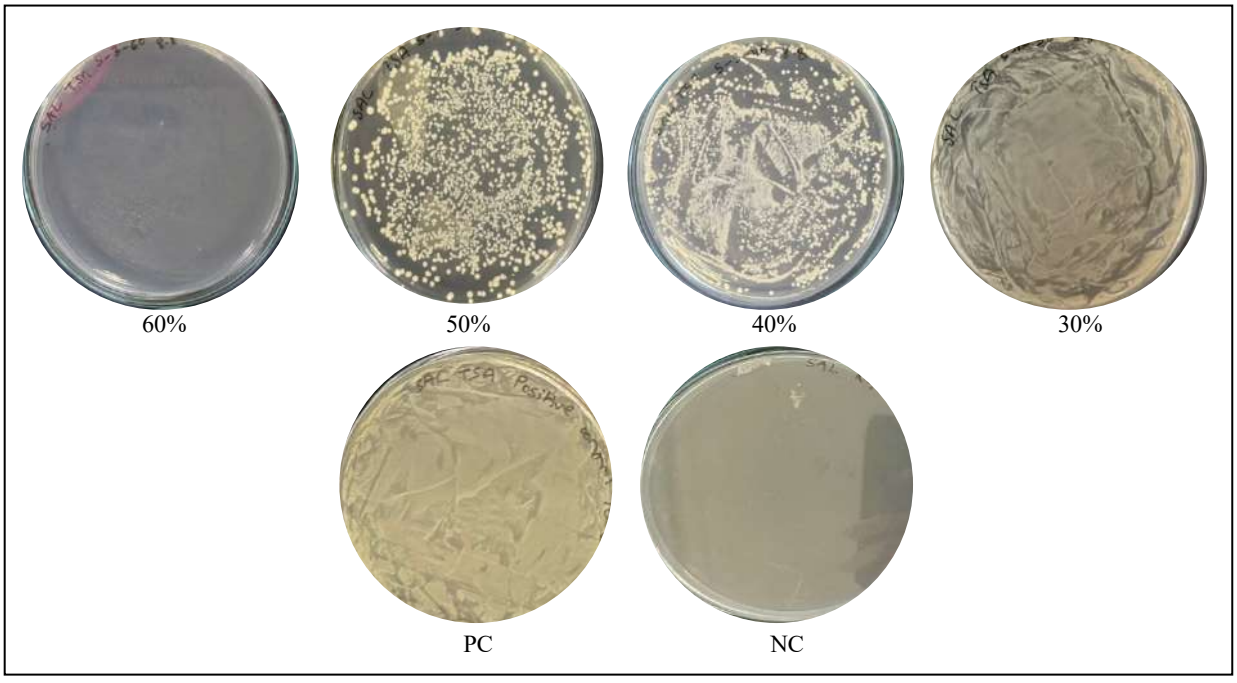
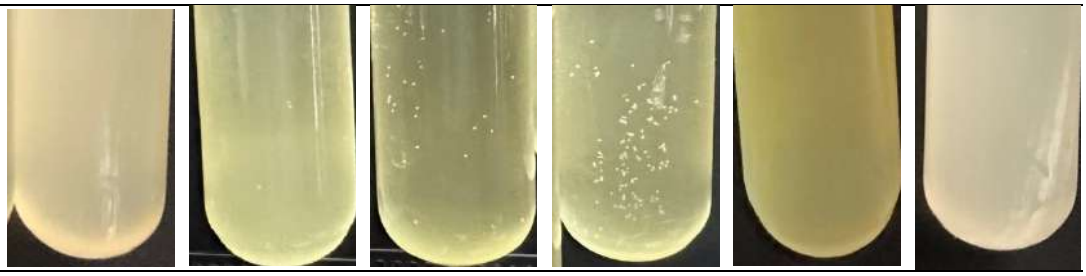


Figure 14: MBC results of Sepnil® against the Sepnil®-adapted strain.

The MBC of Sepnil® against the Sepnil®-adapted strain was determined to be 60% as no bacterial growth was observed on the corresponding plate. The highest concentration of Sepnil® at which bacterial growth was still visible (before reaching MBC) was on the plate

with 50% Sepnil® (v/v), confirming the MIC of Sepnil® as 50%. Thus, the MIC of Sepnil® increased by 1.25-fold in the evolved strain.

Table 13: MIC of Sepnil® against the Sepnil®-adapted strain after 10 d of subculture in Sepnil®-free broth.

Concentration	60%	50%	40%	30%	Positive control	Negative Control
Results						

MIC could not be visually determined due to the nature of Sepnil® (turbidity was observed in the negative control). Hence, MBC was done using these results to confirm the MIC (Figure 15).

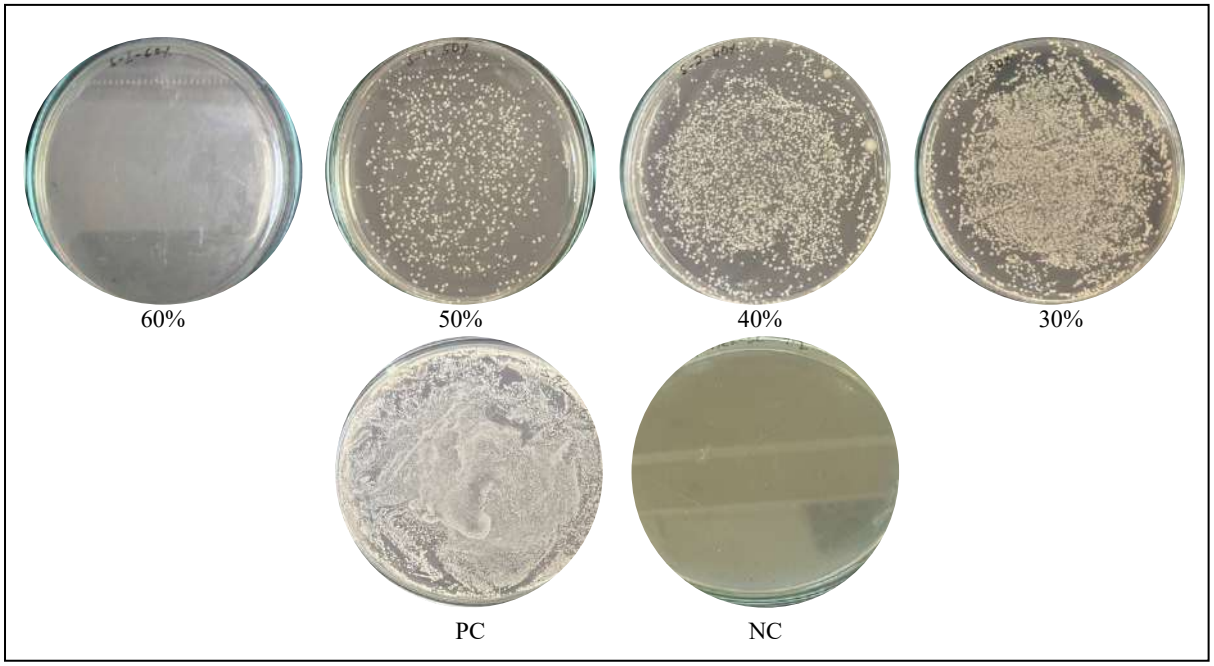


Figure 15: MBC results of Sepnil® against the Sepnil®-adapted strain after 10 d of subculture in Sepnil®-free broth.

As shown in Figure 15, it was determined that the MBC of Sepnil® against the Sepnil®-adapted strain after 10 d of repeated subculture in Sepnil®-free broth was 60% as no bacterial growth was observed on the plate. Thus, the MIC of Sepnil® was confirmed to be 50% (the highest concentration of Sepnil® showing bacterial growth), and the increased tolerance of the Sepnil®-adapted strain against Sepnil® remained stable after 10 d.

3.4.2 Antibiotic Susceptibility Test

Table 14: Resistance pattern of *S. aureus* before and after the adaptation to Sepnil®.

Antibiotic	Zone of inhibition (mm)	
	Parent Strain	Sepnil®-adapted Strain
MRP	38 (S)	38 (S)
AZM	32 (S)	32 (S)
TE	31 (S)	32 (S)
MET	22 (S)	24 (S)
GEN	29 (S)	29 (S)
C	28 (S)	29 (S)
CIP	31 (S)	31 (S)
CD	31 (S)	32 (S)
AK	29 (S)	26 (S)
LZ	32 (S)	32 (S)
CAZ	0 (R)	0 (R)
E	32 (S)	30 (S)

S: susceptible; R: resistant; MRP: meropenem; AZM: azithromycin; TE: tetracycline; MET: methicillin; GEN: gentamycin; C: chloramphenicol; CIP: ciprofloxacin; CD: clindamycin; AK: amikacin; LZ: linezolid; CAZ: Ceftazidime; E: Erythromycin.

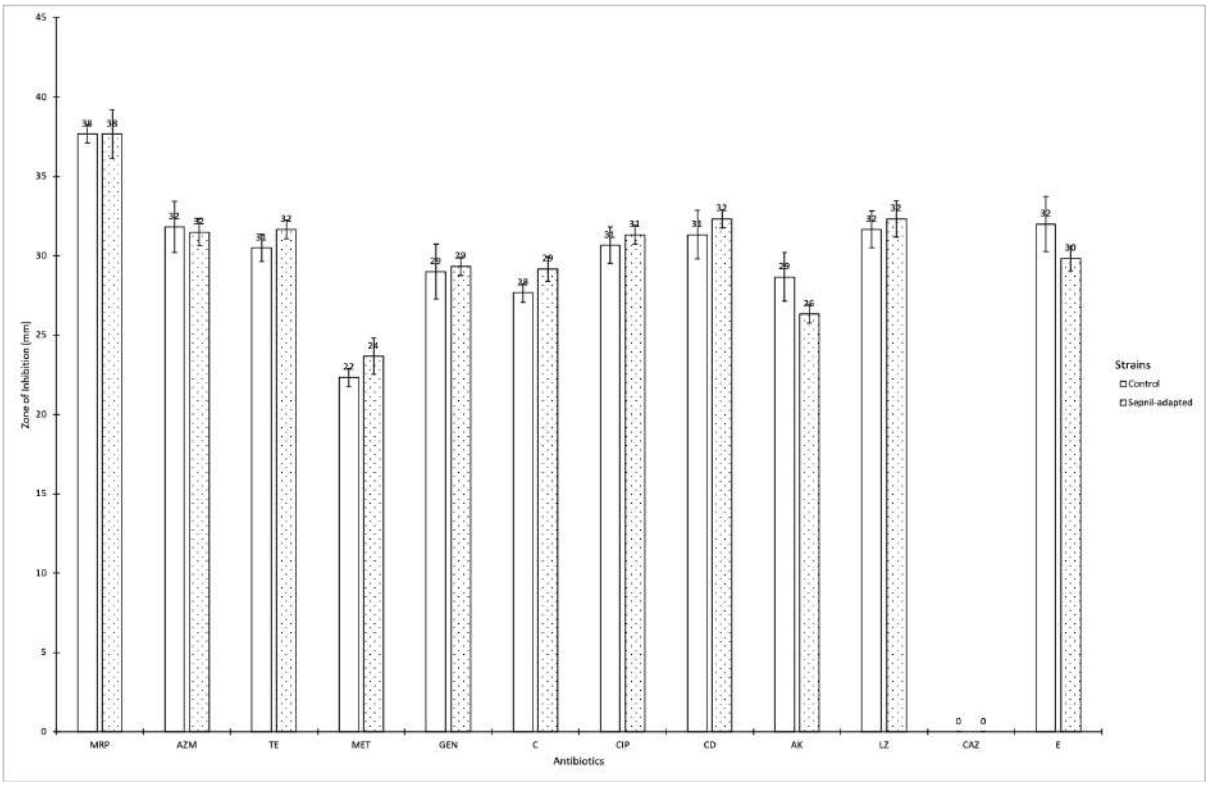


Figure 16: Comparative analysis of the zone of inhibition (mm) measured for the parent strain and Sepnil®-adapted strains against antibiotics. MRP: meropenem; AZM: azithromycin; TE: tetracycline; MET: methicillin; GEN: gentamycin; C: chloramphenicol; CIP: ciprofloxacin; CD: clindamycin; AK: amikacin; LZ: linezolid; CAZ: Ceftazidime; E: Erythromycin.

The data (Table 15, Figure 16) represent the mean zone diameter values obtained from three independent experiments, and the standard deviation of the mean is shown by the vertical error bar. The AST results for the control strain were consistent with those for the parent strain. The Sepnil®-adapted strain remained susceptible to all antibiotics, except ceftazidime, to which it remained resistant. There was an increase in susceptibility to most antibiotics; an overall decrease in sensitivity to amikacin, azithromycin, and erythromycin; and no change in susceptibility to meropenem. However, none of these changes was statistically significant ($p>0.05$).

3.4.3 Growth Curve Assay

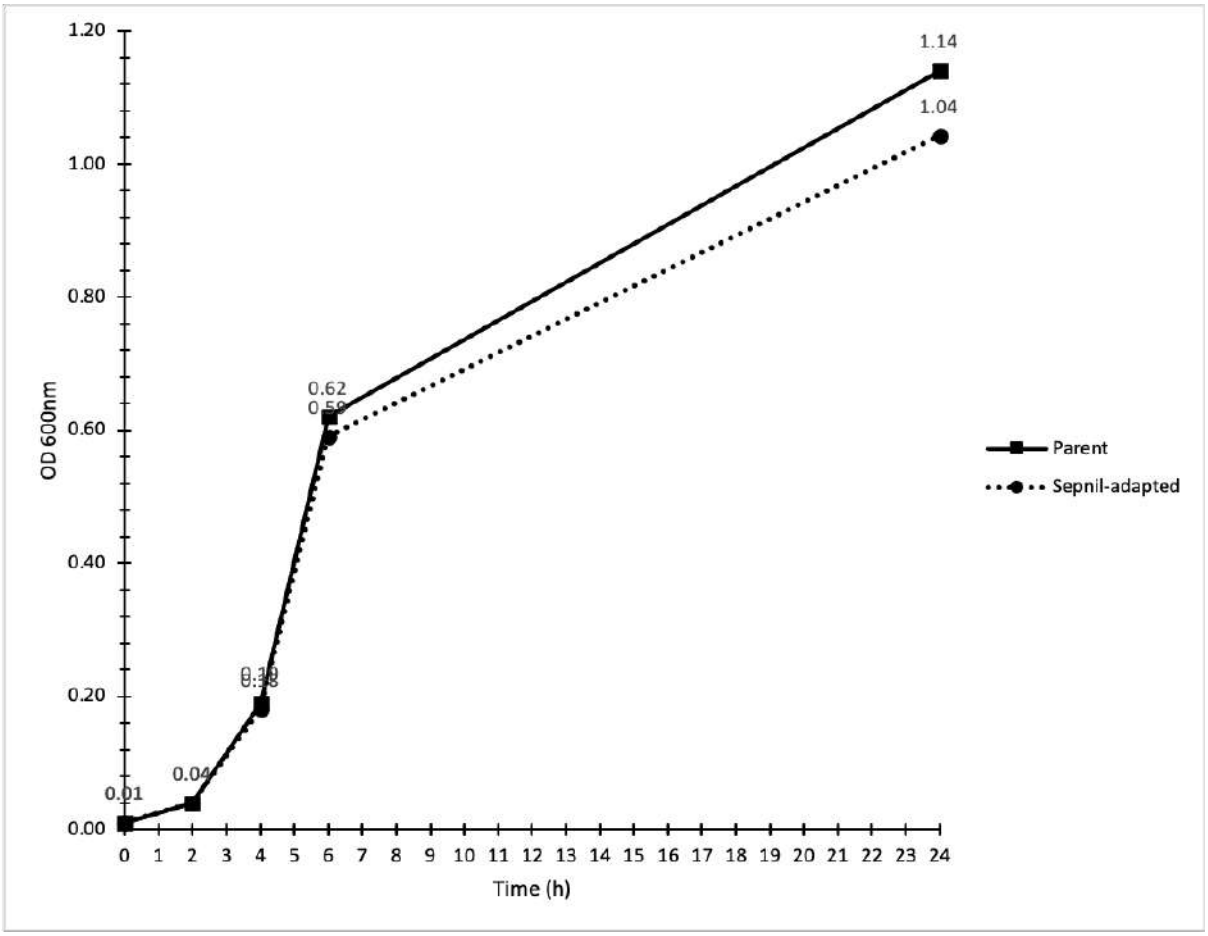


Figure 17: Growth capacity of parent strain versus Sepnil®-adapted strain.

A very similar growth trend was noted between the parent and Sepnil®-adapted strains (Figure 17). The Sepnil®-adapted strain showed slightly lower optical density values (and thus, growth rate). However, there was no statistically significant differences in growth capacity of the parent ($M = 0.4$, $SD = 0.48$) and Sepnil®-adapted strains ($M = 0.42$, $SD = 0.46$), $t(4) = -0.943$, $p = 0.399$.

Chapter 4

Discussion

Concerns over the effectiveness of biocides and the decrease in bacterial susceptibility have arisen from the increased use of disinfectants such as hand sanitisers. There is concern that bacteria, such as *Staphylococcus aureus* strains may develop resistance to common disinfectants. The results include a thorough analysis of the microbiological reactions to the three hand sanitiser formulations most widely used in Bangladesh: Hexisol®, 70% ethanol, and Sepnil®. The sanitisers varied in formulations: Hexisol®- 70% w/w isopropyl alcohol and 0.5% w/v chlorhexidine gluconate; Sepnil®: 70% ethanol along with carbomer, glycerin, polyethylene glycol, TEA, water, and fragrance; and 70% ethanol. In this project, the main topics of investigation were the evolutionary ability of bacteria to adapt to hand sanitisers and the possibility of induced antibiotic resistance. The results showed that different hand sanitiser formulations can result in different evolutionary pathways, some of which promote the emergence of cross-resistance and others which do not. Antibiotic susceptibility, growth characteristics, microbial adaptation, and variations in the MIC (and MBC for Hexisol® and Sepnil®) were evaluated for each formulation. These results provide insights into the dynamic relationships between these widely used hand sanitisers and microorganisms.

4.1 Detection of *mecA* gene

The PCR amplification and subsequent gel electrophoresis revealed the presence of the *mecA* gene in the test sample which means that it is a MRSA strain. The *mecA* gene codes for the production of Penicillin-Binding Protein 2a (PBP2a) (Vannuffel et al., 1995), as a result, bacteria are less susceptible to β-lactam antibiotics such as cefoxitin, oxacillin, and methicillin. However, AST result of the parent strain for Methicillin 5 µg was susceptible. This indicates

that although the strain carried the *mecA* gene, it did not express it under normal conditions and the gene was only expressed when the strain was under selective pressure caused by the Hexisol® (chlorhexidine) exposure. As stated previously, many studies indicate that MRSA is less sensitive to CHX than MSSA; moreover, *qacA/B* is more commonly found in MRSA strains than MSSA (Lu et al., 2015).

4.2 Adaptation of Bacteria to Hand Sanitisers

Based on this study, MRSA strains may become more tolerant to biocides by being exposed to progressively higher concentrations of hand sanitisers. It is noteworthy, however, that there were differences in both the efficacy and degree of MIC increase among the various hand sanitisers; the efficacy variation may have resulted from formulation differences. Among them, Hexisol® showed the most significant change, with a four-fold increase in the MIC in the Hexisol®-adapted strain and this could be due to the presence of chlorhexidine in its formulation; chlorhexidine was not present in Sepnil® or 70% ethanol. Specifically, the MIC of Hexisol® increased from 0.9375% (v/v) to 3.75% after experimental evolution. Despite this increase, the Hexisol® volume required for inhibition remained below the recommended dosage (WHO recommends a dosage of 3ml) suggesting that it is highly efficient in killing bacteria however; it comes with risks of inducing antibiotic resistance (see section 3.2.2). Therefore, according to the definitions in section 1.5.1, the adapted strain developed tolerance after exposure to sub-lethal concentrations of Hexisol®. Given that this hand sanitiser contains chlorhexidine, the acquisition and/or upregulation of multidrug efflux pumps, specifically *qacA/B* may account for the increased tolerance. As stated previously, many studies have reported that the increased tolerance of *S. aureus* (e.g. MRSA) to chlorhexidine is mainly attributed to the presence of closely related plasmid-based *qacA* and *qacB* genes that code for multidrug efflux pump (do Vale et al., 2019). To verify this theory, it is necessary to quantify the RNA expression of *qacA/B* genes during the exposure to Hexisol®. After 10 subcultures in

Hexisol®-free broth, the adaptive resistance of the evolved strain to Hexisol® remained steady, indicating that repeated exposure to Hexisol® can induce stable modifications in the adapted-strain.

In the case of 70% ethanol, the MIC increased from 30% to 40%, representing a 1.33-fold increase. Although the ethanol MIC increase was higher than the recommended dosage, it was not as substantial as the Hexisol® MIC increase. Based on the terminology covered in section 1.5.1, the strain adapted to ethanol showed resistance to 70% ethanol because it could still survive in 40% (v/v) or 4 mL of 70% ethanol. As stated previously, the observed increased tolerance of *S. aureus* to ethanol could be due to an overall upregulation of genes linked to fatty acid metabolism, nucleotide synthesis, energy metabolism, and the robust formation of biofilms (Korem et al., 2010). However, a molecular analysis of the evolved strain is required to pinpoint the exact resistance mechanism. Ten subcultures in ethanol-free broth did not affect the adaptive resistance of the evolved strain to ethanol, indicating that repeated exposure to ethanol could induce stable changes in the adapted strain.

The results of Sepnil®-adapted strains were similar to that of ethanol-adapted strains and this could be due to the similarity in formulation: both of the hand sanitisers consist of 70% ethanol. The MIC of Sepnil® against the MRSA increased. After exposure to a gradually increasing sub-inhibitory concentration of Sepnil®, the MIC of the parent strain increased from 50% (v/v) to 40% (v/v). However, according to the terminologies discussed in section 1.5.1, the parent strain was already resistant to Sepnil®, as it required 40% (v/v) Sepnil® (equivalent to 4 ml) for bacterial inhibition. The poor efficacy of Sepnil® could be due to its glycerol content; as stated previously, glycerol is a highly interfering component where its 3 -OH groups overlap with the ethanol spectrum (Littlejohn et al., 1991). Furthermore, Sepnil® contains several other ingredients, such as perfume, polyethylene glycol, carbomer, and TEA, which may reduce its antimicrobial efficacy. Since Sepnil® also contains 70% ethanol, the adaptive resistance

mechanism is likely similar to that of 70% ethanol. Upon 10 subcultures in Sepnil®-free broth, the evolved strain's adaptive resistance to Sepnil® remained steady, indicating that repeated exposure to Sepnil® can induce stable modifications in the adapted strain.

For all tested hand sanitisers, 12 d of exposure increased bacterial tolerance by 1.25–4 fold, which persisted after 10 subcultures in fresh broth. This increased tolerance to hand sanitisers indicates that bacteria can quickly adapt to disinfectants used in healthcare and households, which can affect public health. The persistence of this increased tolerance after repeated subculturing suggests long-term physiological or genetic changes in the bacteria. These findings highlight the importance of monitoring bacterial responses to disinfectants and re-evaluating hand sanitiser formulations and application protocols.

4.3 Co-Selection of Antibiotic Resistance

Regarding antibiotic susceptibility, the Hexisol®-adapted strain displayed elevated resistance to specific antibiotics (azithromycin, methicillin, and erythromycin), indicating a potential co-selection phenomenon. Methicillin belongs to the β-lactam class, while azithromycin and erythromycin belong to the class of macrolides. As stated in section 1.4.4, previous clinical studies have found higher prevalence of *qacA/B* in MRSA strains compared to MSSA. Methicillin resistance in *Staphylococci* arises from the integration of SCCmec, which carries the *mecA* gene along with other antibiotic resistance genes including resistance to macrolides (Ghaznavi-Rad et al., 2010). Moreover, a genetic linkage has been documented between *qac* genes and erythromycin resistance genes on the same plasmid found in staphylococci (Noguchi et al., 2006). As observed by several clinical studies, a genetic linkage between *qac* genes and resistance to multiple antibiotics indicates that strains containing both biocide and antibiotic resistance genes may have a competitive advantage (Zhang et al., 2011). These reports suggest that *qac* genes in *S. aureus* may result in co-selection in antibiotic-resistant strains. Thus, the co-selection of the Hexisol®-adapted strain to methicillin, azithromycin, and erythromycin

seen in this experiment is consistent with previous research. While the genetic linkage between *qac* genes and genes conferring resistance to β-lactam and erythromycin (Malherbe et al., 2013) has been previously reported, the same has not been done for azithromycin. However, it is essential to conduct further molecular analyses to substantiate these assertions. Interestingly, after subjecting the Hexisol®-adapted strain to 10 subcultures in Hexisol®-free broth, the adaptive co-selection to antibiotics reverted to the original MIC value. This phenotypic instability can be attributed to the overexpression of efflux pumps in the absence of their substrates, which might confer a selective disadvantage. The reversion to the parent strain phenotype in the absence of selective pressure mitigates these potential disadvantages. In contrast, adaptive tolerance to Hexisol® remained stable after 10 subcultures in Hexisol®-free broth, while adaptive resistance to antibiotics did not; this supports the hypothesis that the primary mechanism employed by the Hexisol®-adapted MRSA predominantly involves co-resistance. If the Hexisol®-adapted strain had acquired "cross-resistance" to antibiotics, the Hexisol®-induced antibiotic resistance would have remained stable due to a shared mechanism for their elimination.

Furthermore, a statistically significant increase in susceptibility to several antibiotics was observed for the Hexisol®- and ethanol-adapted strains. Similar results for benzalkonium chloride-tolerant strains was also described in a study, which showed increased sensitivity to amikacin, gentamicin and tobramycin (Joynson et al., 2002). A study by Gadea et al. (2017) found higher sensitivity to both antibiotics and biocides following exposure to hexadecylpyridinium chloride as well as benzalkonium chloride in evolved strains. However, the underlying mechanism for this phenomenon remains unexplored. Although increased susceptibility to some antibiotics to which the strain was previously susceptible was observed, susceptibility to ceftazidime, an antibiotic to which the strain had previously been resistant,

was not restored. Moreover, the Sepnil®- nor ethanol-adapted strains demonstrated co-selection against antibiotics.

4.4 Growth Capacity

In accordance with other studies performed with biocide-adapted bacterial strains (Deptuła & Gospodarek, 2010; Gilbert & McBain, 2003), The growth curve assay revealed a significant decrease in bacterial growth rate for the Hexisol®-adapted strain compared to the parent strain. This underscores the existence of a potential trade-off between heightened Hexisol® tolerance and growth efficiency, signifying that bacteria may have modified its physiological characteristics to contend with the stress induced by Hexisol®. This observation was in accordance with another study performed with biocide-adapted bacterial strains (Deptuła & Gospodarek, 2010; Gilbert & McBain, 2003) that stated that bacterial adaptation to biocides and antibiotics comes with fitness costs.

Nonetheless, there was no significant alteration in the growth capacity of the Sepnil®- or ethanol-adapted strains compared to the parent strain. This suggests that these adaptations did not entail analogous fitness costs as those observed in the Hexisol®-adapted strain.

4.5 Limitations of Research and Future Prospects

Although this study aimed to thoroughly understand how bacteria adapt to hand sanitisers, some limitations were noted. To enable a direct comparison between hand sanitisers and control conditions, this study focused on a single species of bacteria in a controlled laboratory environment. It may be necessary to conduct additional investigations to determine whether these results can be applied to other bacterial species. Acknowledging the possibility that resistance in bacterial populations arises and persists owing to different mechanisms is critical. For example, in an uncontrolled environment, resistance develops mainly because of the horizontal transfer of genetic elements within mixed populations (Merchel Piovesan Pereira et

al., 2021). Utilising an experimental evolution study in a heterogeneous bacterial population may provide important new information about the intra- and inter-species transfer of genes resistant to biocides.

Furthermore, because of resource limitations, whole-genome sequencing and RT-qPCR methods were not used to evaluate the expression of resistance genes or to identify putative mutations in adapted strains. In particular, the potential link between phenotypic chlorhexidine tolerance and the presence of *qacA/B* genes could not be investigated. Whole-genome sequencing would make finding mutations linked to porins and multidrug efflux proteins easier, clarifying the mechanisms underlying the (co-)resistance that results. Furthermore, quantifying the expression of resistance genes, such as *qacA/B, mecA, ermA, ermB, ermC, msrA*, and *mef,* in the parent and evolved strains using RT-qPCR would provide insight into the complex process of bacterial adaptation that results in increased biocide tolerance and co-selection against various antibiotic classes.

This study evaluated in vitro adaptation; therefore, the application of these results to in vivo conditions should be carefully considered. Geographic differences in the prevalence of genes that confer resistance to chlorhexidine may be attributed to several factors, such as the dissemination of clones, clinical and demographic makeup of the population being studied, different approaches to infection control, and extent of chlorhexidine administration (Horner et al., 2012). In Bangladesh, the prevalence of reduced susceptibility to CHX among *S. aureus* strains is unknown. Therefore, to contribute to our understanding of this phenomenon, extensive multicentre studies that include clinical and environmental isolates from this region are necessary.

Chapter 6

Conclusion

Growing concerns about the increased use of hand sanitisers and their possible effects on biocide efficacy and bacterial susceptibility are addressed in this study. The investigation focused on three widely used hand sanitisers in the BRAC University Laboratory: Hexisol®, 70% ethanol, and Sepnil®. This study utilised an experimental evolution design to examine how these sanitisers influence bacterial responses, providing insight into the emergence of co-selection to antibiotics and differences in microbial adaptation.

The results showed that when exposed to sub-inhibitory concentrations of hand sanitisers, MRSA strains may become more tolerant. Notably, there were differences in tolerance among the various sanitisers, which may be due to differences in formulation. For all tested hand sanitisers, 12 d of exposure increased bacterial tolerance by 1.25–4 fold, which persisted after 10 subcultures in fresh broth. This decreased susceptibility to hand sanitisers suggests that bacteria can quickly adapt to disinfectants, which may affect public health. The Hexisol®-adapted strain exhibited co-selection with methicillin, azithromycin, and erythromycin among other antibiotics. This co-selection is consistent with previous research, which emphasises the intricate connection between biocide exposure and the development of antibiotic resistance. Growth rate assays of the Hexisol®-adapted strain showed a significant drop in bacterial growth, suggesting a possible trade-off between increased Hexisol® tolerance and growth efficiency. Consistent with earlier research, this observation highlights the fitness costs incurred by bacteria when adapting to biocides.

Although the study offers valuable insights into how bacteria adapt to hand sanitisers, it is important to recognise that it has certain limitations. Further research is required to determine

the in vivo applicability of this research because the focus is on a single species of bacteria in a controlled laboratory setting. Furthermore, quantifying resistance gene expression is essential to comprehending the complex bacterial adaptation responses.

In conclusion, this study highlights the dynamic relationship between bacterial adaptation to hand sanitisers and underscores the importance of maintaining ongoing surveillance of bacterial responses to disinfectants. Given these findings, it is prudent to reassess the use of biocidal agents as hand sanitisers, favouring those with lower or no potential for inducing antimicrobial tolerance or resistance. Hand sanitisers are the primary choice for both healthcare practitioners and the general public to mitigate healthcare-associated infections (Kampf, 2016). The incorporation of supplementary biocidal agents, such as chlorhexidine digluconate or octenidine, into alcohol-based hand rubs may not be imperative (Leaper & Edmiston, 2017). Therefore, it is crucial to review the formulation of sanitisers. If hand sanitisers containing CHX are regularly employed for hand hygiene, they may constitute a significant source of overall CHX exposure. A notable concern with CHX exposure is the sustained selection pressure that can foster resistance to CHX, potentially extending resistance against antibiotics (Septimus & Schweizer, 2016). Consequently, preference should be given to products devoid of additional biocidal agents, provided they exhibit equivalent user acceptability and efficacy for hand disinfection—a practice akin to "antiseptic stewardship" (Kampf, 2018).

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