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Control Strategies of *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* (MRSA) Biofilms: A Review

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ABSTRACT

Staphylococcus aureus is an opportunistic pathogen infecting the human respiratory tract, nasal areas, and skin. In contrast, methicillin-resistant *Staphylococcus aureus* (MRSA) is more pathogenic than *S. aureus* due to its multiple antibiotic resistance. Both *S. aureus* and MRSA can form biofilm and severely threaten public health worldwide. This review discusses potential strategies to prevent or control biofilm formation by *S. aureus* and MRSA, including disinfectants, bioactive glasses, antibiotics, drugs, medicinal plants, nanoparticles, bacteriophages, phytochemicals, and antimicrobial coating. Advanced instrumentations such as Fluorescence Microscopy (FM), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and Fourier-Transform Infrared (FTIR) spectroscopy are helpful to evaluate the efficacy of different strategies used to intervene in *S. aureus* and MRSA biofilms.

Keywords: *Staphylococcus aureus, methicillin-resistant Staphylococcus aureus (MRSA), biofilm control*

INTRODUCTION

This prevention of nosocomial infections has become the primary concern in the healthcare industry. One of the major nosocomial pathogens is a bacterial species known as *Staphylococcus aureus* or methicillin-resistant *Staphylococcus aureus* (MRSA) [1]. MRSA is more pathogenic than *S. aureus* as it develops resistance to antibiotics. Over the last few decades, there has been an upsurge in MRSA cases worldwide [2]. Due to the recent Coronavirus disease 2019 (COVID-19) pandemic, nosocomial MRSA cases have increased substantially [3]. Skin and soft tissue infections occurring in the emergency department are commonly caused by MRSA [4]. Infected individuals often suffered financial burdens as the infection prolonged their hospital stay [5].

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S. aureus can cause chronic infections as biofilm formation allows it to resist treatment [6]. This has dramatically increased the novel study of drugs and vaccines against the pathogen [7]. Biofilm-forming mutants of MRSA infections are often very difficult to cure. The extracellular matrix is known to enhance the ability of *S. aureus* to acquire resistance against wide-spectrum antibiotics, including methicillin, thereby making the conventional antibiotic treatment ineffective. The emergence of biocide resistances and the high environmental impact of disinfectants currently applied in the food industry have led to the search for novel antimicrobial compounds and develop innovative sanitizing procedures to control undesirable microorganisms such as *S. aureus* [8]. The *S. aureus* and MRSA biofilm control strategies need to be better documented. Thus, this review highlighted the strategies for *S. aureus* and MRSA biofilm control.

Staphylococcus aureus and Methicillin-Resistant *Staphylococcus aureus*

S. aureus is the primary human pathogen that induces various medical manifestations. *S. aureus* is a Gram-positive bacterium commonly found in normal human flora and the surrounding environment. *S. aureus* often colonizes the skin and mucous membranes of most healthy individuals. However, this bacterium does not usually cause infection unless it penetrates the internal tissues or enters the bloodstream of individuals [9]. While it is known to cause skin and soft tissue infections, it can also potentially infect almost any organ system in the human body, leading to fatalities. It can also cause other infections, such as bacteremia, pneumonia, skin infections, and food poisoning [10].

Moreover, the ability to possess a wide array of virulence factors makes *S. aureus* a successful pathogen causing various human and animal infections. These virulence factors assist *S. aureus* in attaching to their host, destroy the host's immune defense and invade host tissue, resulting in septicemia and toxic shock syndrome (TSS). The treatment for *S. aureus* remains a significant concern and challenge before the emergence of multi-drug resistant strains such as Methicillin Resistant *S. aureus* (MRSA). MRSA is different from other *Staphylococcus* bacteria since it cannot be eliminated by antibiotic methicillin and other related medications. This has led to higher mortality rates, increased morbidity, and a rise in the cost of healthcare treatment. Over the last few decades, *S. aureus* has been known as a potent biofilm producer in medical devices and the host tissue surfaces.

Biofilm and its Formation

Biofilm is a microbial community that lives together to form a hydrated mat-like structure surrounded by an extracellular matrix that protects from host immune response, antimicrobial treatments, and antibiotic diffusion within the biofilm matrix [11, 12]. It is challenging to eliminate biofilm due to the bacterial tolerance towards removing free-floating and planktonic bacteria by antimicrobials concentrations during biofilm growth [13]. Previous works on various bacterial species forming the biofilm have identified that the biofilm life cycle can be understood in four steps: reversible adhesion, irreversible attachment, maturation, and dispersion, as shown in Figure 1 [14]. In brief, stress response impulse and the attachment of bacteria on a surface with high cell proliferation initiates the biofilm life cycle. Monolayer is then formed, and the expression of several specific genes triggers the microcolonies formation. A well-organized biofilm structure is developed with the help of a quorum-sensing signaling system. Quorum sensing (QS) is a mechanism where bacteria cells respond and synthesize a variety of signaling molecules to allow communication with each other cells [15]. The quorum-sensing system is the most studied in *S. aureus*.

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The expression of *S. aureus* colonization factors is activated during dispersion and terminated due to the suppressed quorum-sensing system [14].

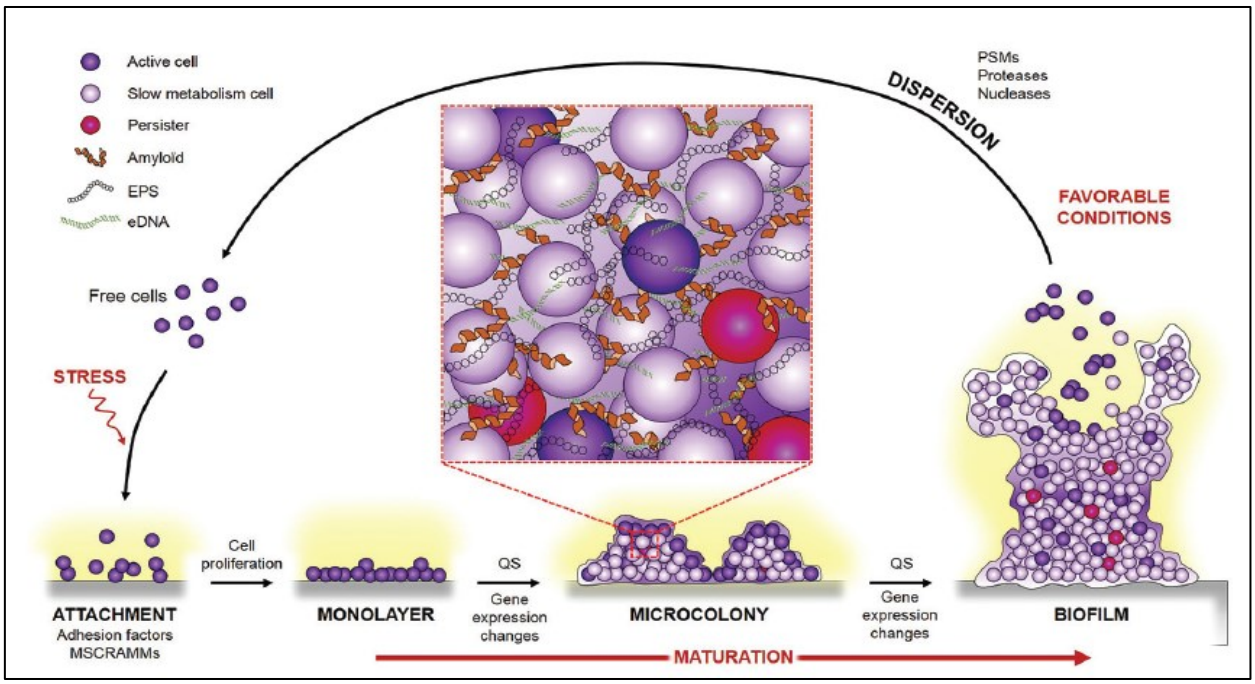

Figure 1: Life cycle of biofilm [14]

The biofilm life cycle begins with the reversible adhesion of free-floating and planktonic bacteria to a surface depending on the viral gene products, bacterial species, environmental factors, and composition of the surface [14, 15]. In this case, the bacteria are susceptible to gentle cleansing, antibiotic treatment, or altered conditions [15]. The preferred surface includes parameters such as hydrophobic, rough, coated with a conditioning film made of polymers, with high cation and concentration of nutrients.

During irreversible attachment, microcolonies are formed by aggregation of cells, and cell division occurs by creating more adhesion sites to allow the recruitment of other cells [15]. The cell clusters mature, form a thick layer, and embed in the extracellular matrix [16]. Biofilms then mature and become heterogeneous when microcolonies reach their maximum thickness [17 - 20]. The multicellular structure development promotes biofilm maturation whereby the bacteria produce a matrix to create an intercellular aggregation so that the bacterial cells stick to each other and on the surface [14]. The composition of the matrix includes exopolysaccharides (EPS), proteins, and extracellular DNA (eDNA). In *S. aureus*, specific proteins, which are surface protein G (SasG) and surface protein C (SasC), could be substituted for EPS-like polysaccharide intercellular adhesion (PIA). Maturation of biofilm and intercellular aggregation is possible because the SasC possesses an LPXTF motif, which helps with its attachment to the cell wall.

The life cycle of biofilms ends with mature biofilms detaching small segments from them to allow their propagation to other sites or the release of planktonic bacteria [15]. The disrupted wall of the

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microcolony allows the cells to escape from the biofilm and become single bacteria by entering the bulk liquid [14]. The biofilm is left with central voids or may further rupture if the response of dispersion is extensive. In *S. aureus*, the effect of dispersal was also found to be linked to Agr quorum-sensing.

Strategies for Staphylococcus aureus Biofilm Control

The research on biofilms and strategies to significantly reduce them are among today's most critical research areas. As biofilm formation corresponds to bacterial pathogenicity and the spread of antibiotic resistance, some strategies need to be implemented to address this issue [9]. Numerous reviews of antibiofilm have already been carried out, but the present review focuses specifically on different strategies for biofilm control against *S. aureus* bacteria. These strategies include using antibiotics, medicinal plants, nanoparticles, disinfectants, bacteriophages, bioactive glasses, drugs, purified phytochemicals, and antimicrobial coatings. Table 1 shows the summary of anti-biofilm strategies.

Antibiotics

S. aureus biofilm can withstand antibiotic treatments and survive extreme environments by weakened penetration and disrupting the host immune response, which leads to infections [21]. The rise of multidrug-resistant strains of *S. aureus* has become the current health problem [22]. The activities of moxifloxacin, rifampin, vancomycin, and combining 4-ethoxy benzoic acid (4EB) with vancomycin were demonstrated in *S. aureus* biofilm. Desrosiers *et al.* [23] studied the topical antibiotic moxifloxacin *in vitro* activity against clinically isolated *S. aureus* biofilm. They found a 2-2.5 log decrease in viable bacteria when *S. aureus* biofilm was treated with moxifloxacin at 1000 × (0.1-0.2 mg/mL). Furthermore, Douthit *et al.* [24] demonstrated the ability of powdered vancomycin and rifampin to prevent and eliminate *in vitro S. aureus* biofilm on stainless-steel implants. It was proven that vancomycin, rifampin, and the combination of both successfully inhibited and destroyed the production of *S. aureus* biofilm.

A different study was conducted by Campbell *et al.* [25] to reduce antibiotic resistance in ciprofloxacin-resistant methicillin-resistant *S. aureus* (CR-MRSA) strains were found to be a promising approach. The optimization of NCE formulations was characterized by scanning electron microscopy (SEM), dynamic light scattering (DLS), and transmission electron microscopy (TEM). Figure 2 shows the morphological identification of NCE formulation for SEM and TEM. The antibacterial activity by NCE towards CR-MRSA biofilm is shown by the reduced value of MBIC and the prevention of biofilm formation by reversing the expression of gene *icaB* involved in forming a biofilm.

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Table 1: Summary of antibiofilm strategies against *S. aureus* and MRSA

Types	Strains	Examples	References
Antibiotics	*S. aureus*	Moxifloxacin, rifampin, vancomycin, and 4-ethoxybenzoic acid (4EB) combined with vancomycin.	[23, 24, 25]
	Ciprofloxacin-resistant methicillin-resistant *S. aureus* (CR-MRSA)	Niosomes encapsulated ciprofloxacin (NCE).	[27]
Medicinal plants	*S. aureus*	Essential oil extracted from *Thymus vulgaris, Lippia sidoides* and *Pimenta pseudochariophyllus.*	[8]
	MRSA	*Cochlospermum regium, Sapindus mukorossi,* and the essential oil extracted from *Amomum villosum* Lour.	[11, 12, 28]
Nanoparticles	*S. aureus*	Curcumin (Cur) loaded on positively charged chitosan nanoparticles (CSNP) and a silver salt of 12-tungstophosphoric acid ($Ag_3PW_{12}O_{40}$) nanoparticles (AgWPA-NPs).	[31, 32]
	MRSA	Pancreatin enzyme (PK) doped on Zinc oxide nanoparticles (ZnONPs) and nano-formulated antibiotics (linezolid and rifampicin).	[33, 34, 35]
Disinfectants	*S. aureus*	Combination of ultrasound and acidic electrolyzed water (AEW) and hydrogen peroxide.	[36 - 38]
Bacteriophages	*S. aureus*	ME18, ME126, SAP26, combination of DRA88 with phage K, and LysCSA13.	[39 - 42]
	MRSA	Combination of DRA88 with phage K, and LysCSA13.	[41, 42]
Bioactive glasses	*S. aureus*	F18 and S53P4.	[43, 44]
Drugs	*S. aureus*	C-10 massoialactone.	[46]
	MRSA	Ibuprofen	[46]
Purified phytochemicals	MRSA	Citral, baicalein, and the combination of baicalein with linezolid.	[26, 47, 49]
Antimicrobial coatings	MRSA	Combination of GOX and AGXX.	[50]

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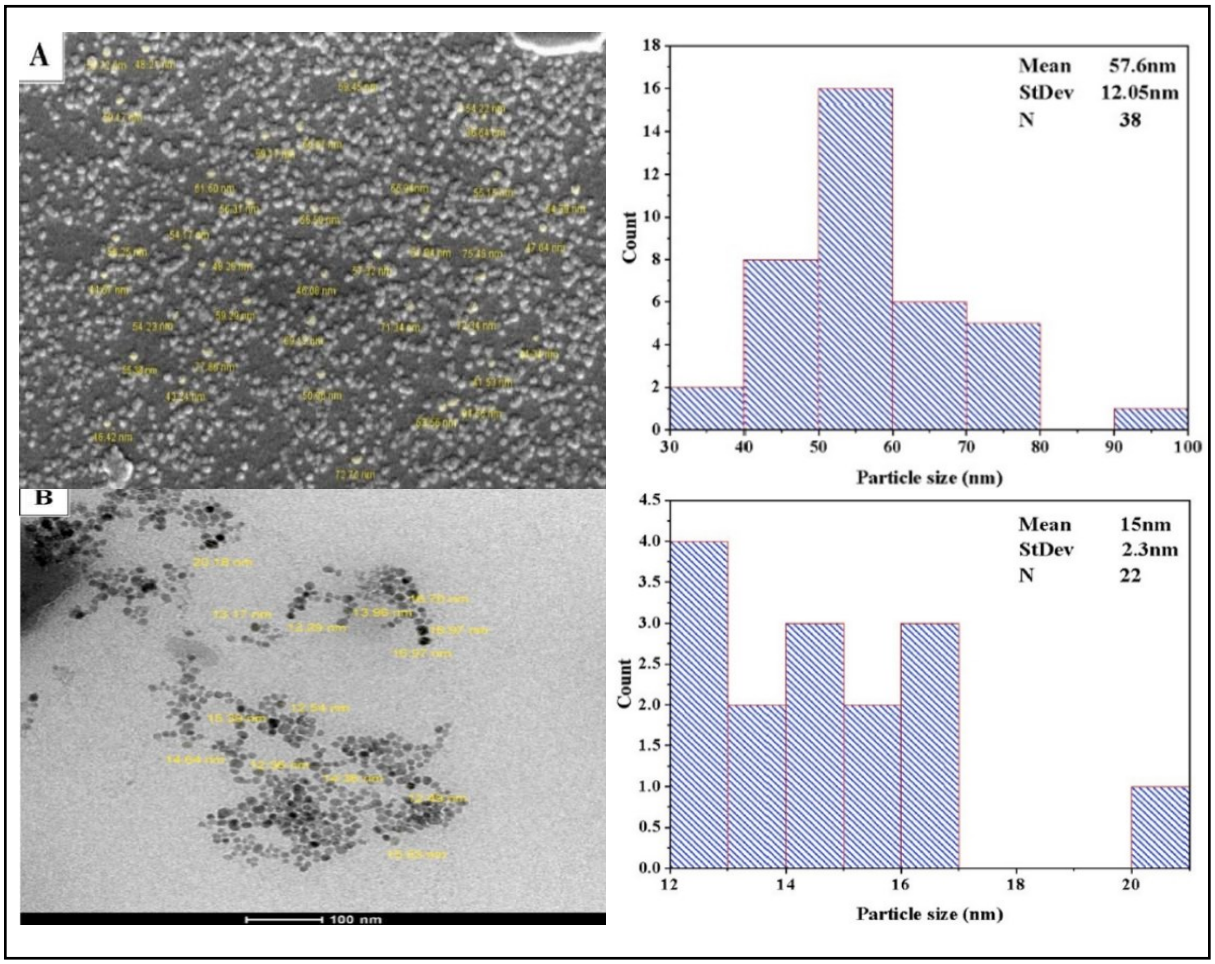

Figure 2: Morphological evaluation of niosomes ciprofloxacin encapsulated (NCE) formulation: (A) Scanning electron microscopy (SEM), (B) Transmission electron microscopy (TEM) [27]

Medicinal Plants

It has been established that various bioactive compounds produced in plants using various secondary metabolic pathways possess antibiofilm properties [11]. In a study by Selvaraj *et al.* [12], the methanolic extract of *Sapindus mukorossi* substantially inhibited MRSA biofilm by 82%, while *in vitro* assays revealed its potential to reduce slime, hydrophobicity of the cell surface, synthesis of EPS, and eDNA, and auto-aggregation. Moreover, oleic acid was associated with antibiofilm activity, as shown by gas chromatography-mass spectrometry (GC-MS) and molecular docking analysis. The presence of oleic acid in the methanolic extract of *Sapindus mukorossi* is further confirmed by Fourier-transform infrared (FTIR) spectroscopy analysis. Figure 3 shows the FTIR spectra of standard oleic acid and methanolic extract of *Sapindus mukorossi.*

Secondary plant metabolites such as essential oil (EO) also possess antibacterial, analgesic, and anti-inflammatory properties [28]. Vázquez-Sánchez *et al.* [8] elucidated the effectiveness and more

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environmentally friendly way by single and combined use of essential oil-based treatments using *Thymus vulgaris, Lippia sidoides,* and *Pimenta pseudochariophyllus* against *S. aureus* biofilms formed on polystyrene and stainless-steel materials in the food industry. The antibiofilm potential of the treatment is due to the presence of thymol, chavicol, and carvacrol and the synergistic effect of other compounds like limonene, p- cymene, and α-pinene in high concentrations. In a different study by Tang *et al.* [28], EO from *Amomum villosum* Lour managed to damage the cell membrane of MRSA, which leads to the prevention of biofilm and protein synthesis, rupture of membrane integrity, and leaked intracellular macromolecular substances.

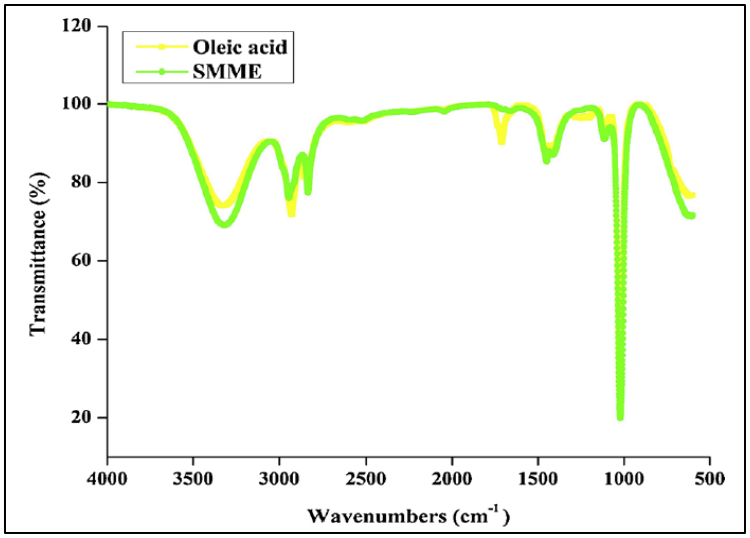

Figure 3: FTIR spectra of standard oleic acid and methanolic extract of *Sapindus mukorossi* [12]

Nanoparticles

The application of nanoparticles for biofilm control has received significant attention due to their versatility, stable temperature, low cost, physical and chemical properties, and large surface area-to-volume ratio [29]. Nanoparticles have been shown to easily penetrate harmful bacterial cells because of their small size [30]. Ma *et al.* [31] investigated the effect of curcumin (Cur), loaded on positively charged chitosan nanoparticles (CSNP), against *S. aureus* biofilm. The SEM and confocal laser scanning microscopy (CLSM) showed the potential of CSNP-Cur in decreasing the biofilm thickness and eliminating the biofilm cells on the silicone surfaces. Meanwhile, Liang *et al.* [32] demonstrated the positive effect of the silver salt of 12-tungstophosphoric acid ($Ag_3PW_{12}O_{40}$) nanoparticles (AgWPA-NPs) in damaging *S. aureus* cell membrane structure and down-regulated its biofilm-related genes Additionally, *S. aureus* biofilm formation was suppressed by AgWPA-NPs as shown by the fluorescence microscopy (FM) analysis.

In 2020, Banerjee *et al.* [33] evaluated the antibiofilm activity of pancreatin enzyme (PK) doped on Zinc oxide nanoparticles (ZnONPs). The anti-biofilms, anti-motility, anti-virulence, and antibacterial properties of ZnONPs-PK against MRSA were more effective than treatment using only PK or ZnONPs. In addition, ZnONPs-PK increased the sensitivity of MRSA towards vancomycin and induced oxidative

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damage to the cell membrane of MRSA. On the other hand, the use of nanoantibiotics in targeting MRSA reservoirs in osteomyelitis patients' bones and its biofilm has previously been reported by Guo *et al*. [34] and Guo *et al*. [35]. The strategy to manipulate the interaction between nanoparticle and biofilm and the interaction between nanoparticle and host cell using nanoformulation of antibiotics (linezolid and rifampicin) can be developed as an alternative therapy to combat MRSA-mediated osteomyelitis.

Disinfectants

Biofilm formation of foodborne pathogens on food production surfaces has resulted in many disease outbreaks and recalls. Disinfectants are the critical intervention methods against infectious organisms on the surface of medical facilities and are used widely as antimicrobials [36]. Shao *et al.* [37] researched removing *S. aureus* biofilm on steel surfaces using ultrasound and disinfectants. The combinations of ultrasound and disinfectants, especially acidic electrolyzed water (AEW), were proven to rapidly inactivate and eliminate *S. aureus*. The use of ultrasound in this removal process was to disrupt or destroy the biofilm matrix rather than sterilize the cells in the biofilm, and the bactericidal result was primarily due to AEW rather than ultrasound.

Healthcare-associated infections (HAIs) are a global challenge to public health, contributing to increased mortality, morbidity, and cost; thus, using such disinfectants is critical to avoid the spread of infectious diseases from infected environments and medical equipment to patients. A typical antimicrobial used to disinfect surfaces in hospitals is hydrogen peroxide. A study by Lineback *et al.* [36] reported the effectiveness of hydrogen peroxide against *S. aureus* biofilms. Hydrogen peroxide disinfectants were observed to break both the biofilm matrix and the bacterial cells, making them antibiofilm solid agents. Furthermore, another research conducted by Köse *et al.* [38] also reported the effectiveness of using hydrogen peroxide compared to other disinfectants.

Bacteriophages

Milk products are believed to be possible sources of zoonotic foodborne pathogens [39]. *S. aureus* is an evolving pathogen from the mammary glands of dairy animals. Since antibiotics are not permitted in food products, the Food and Drug Administration has established natural, safe antimicrobial agents called bacteriophages that can be used at various stages of food processing to improve product safety. Mohamed *et al.* [39] experimented on ME18 and ME126 phages belonging to the Myoviridae family with significantly reduced icosahedral heads and long contractile tails. The experiment demonstrates the ability of phages to be used as antimicrobials in food and sanitizers on machinery in the food industries to destroy bacteria and suppress or eliminate biofilms. The capability of phages to destroy biofilms formed by *S. aureus* bacteria may be correlated to phage-associated polysaccharide enzymes that degrade the EPS.

Other than that, induced phage, SAP26, was derived from the *S. aureus* clinical isolate and studied by Rahman *et al.* [40] as therapies for anti-biofilm. Phage SAP26 displayed a wide variety of lytic activity against *S. aureus*. In addition, combination treatment with phage and antimicrobial agents had an apparent biofilm elimination effect, leading to structural alterations in the biofilm matrix and a significant reduction in the number of bacteria. Both phage and antibiotics could penetrate many biofilms and the cell's basal layer, leading to cell death. From these experiments, they reported that combination therapy of phage SAP-

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26 with rifampicin efficiently disrupted the biofilm matrix and destroyed the bacterial cells. A study by Alves *et al.* [41] mixed a newly isolated phage DRA88 with phage K into a phage mixture and demonstrated that 74% of its lytic potential increased. Using a phage mixture is significantly favored over a single phage as it results in a reduced rate of antibacterial activity. This study provides a viable solution to antibiotic or antimicrobial treatment in the battle against certain *S. aureus* infections and destructive consequences of MRSA infections and associated biofilms, including catheter infections and burn wounds.

On the other hand, on different surfaces, including polystyrene, glass, and stainless steel, a high efficacy of LysCSA13 in removing Staphylococcal biofilms has been reported [42]. A viable cell count assay results show that LysCSA13 decreases the number of sessile cells, and a decrease in the biofilm mass is between 80% and 90%. Additionally, field emission scanning electron microscopy (FESEM) analysis further confirmed the biofilm reduction ability of LysCSA13. The antibiofilm activity of LysCSA13 on stainless steel, glass, and polystyrene surfaces was vigorous. In addition, LysCSA13 showed a significant destroying activity against *S. aureus* biofilms and MRSA strains. The result of the study demonstrates the possible use of LysCSA13 as an effective agent for biofilm control in various processes and environments for food processing [42].

Bioactive Glass

Bioactive glasses are commonly reported to facilitate chemical bonding between the implanted material and the host tissue [43]. These materials induce various biological reactions when in contact with physiological fluids and have benefits that surpass bone regeneration, such as bacterial properties. Passos *et al.* [43] confirmed that F18 bioactive glass particles have effectively suppressed the formation of *S. aureus* biofilm upon direct contact between the inoculum and the biomaterial for 6 hours, reducing approximately 6 logs in the viable bacterial population. An underlying relationship between antimicrobial activity and increased pH has also been observed. Relating to the results obtained, the pH neutralization of the solution with the F18 dissolution products had a good influence on reducing the bioactive glass of the bactericidal activity in *S. aureus* biofilms. Therefore, the F18 is a potential biomaterial for preventing and controlling *S. aureus* infections.

Other findings by Grønseth *et al.* [44] showed that the bioactive glass S53P4 in contact with biofilm destroyed all viable bacteria at the lowest concentration and shortest priming time. This suggests that S53P4 is a potent antibiofilm material with significant antimicrobial effects on *S. aureus* in planktonic and biofilm states. However, the elimination mechanism is not well described. A finding by Coraça-Huber *et al.* [45] also showed that the bioactive glass S53P4 has an apparent growth inhibitory effect on *S. aureus* biofilms. S53P4 can suppress *S. aureus* biofilm formation on titanium discs in vitro. The suppression rate of biofilm cells by S53P4 <45 µm is more efficient against biofilm production in-vitro comparing S53P4 0.5-0.8mm. Therefore, S53P4 seem to have the potential to resolve complication in joint replacement surgeries and the treatment of chronic osteomyelitis.

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Drugs

Since MRSA resists antibiotic treatment, other nonantibiotic drugs have been tested for antimicrobial activity against *S. aureus*. For instance, ibuprofen can cause cytoplasmic membrane destabilization by enhancing cell permeability towards propidium iodide (PI) and releasing intracellular potassium K^+ [46]. Moreover, ibuprofen was able to control biofilm ability through metabolic inactivation and reducing the colony-forming unit (CFU) of *S. aureus*. Natural drugs from essential oils, such as C-10 massoialactone, have potent antibacterial activity against *S. aureus* and can destroy the biofilm [47]. Despite having lower activity than the control chloramphenicol, antibacterial activity can be improved with a higher concentration of C-10 massoialactone. C-10 massoialactone can penetrate the biofilm by dissolving the lipids in the matrix. This action damages the cytoplasmic membrane and membrane protein of *S. aureus,* as shown in Figure 4. On the other hand, Figure 5 shows the image of biofilm before and after being treated with C-10 massoialactone compound under SEM.

Purified Phytochemicals

A study by Padilha da Silva *et al.* [48] found that citral could inhibit MRSA biofilm formation by targeting various virulence pathways. It regulated the expression of CodY, which is vital in repressing virulence pathways such as hemolysis, staphyloxanthin production, and capsular polysaccharide synthesis. The antibiofilm potential of citral was improved by adding an organosulfur compound. The compound synthesized 3-(p-chlorophenyl) thio citronellal showed higher bactericidal activity than citral in controlling MRSA biofilm.

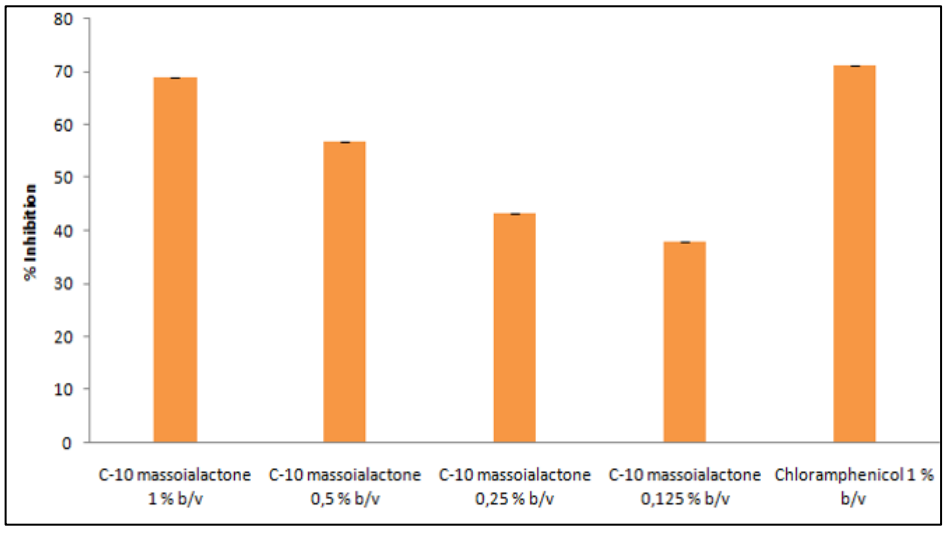

Figure 4: The antibiofilm activity of C-10 massoilactone against *Staphylococcus aureus* [42]

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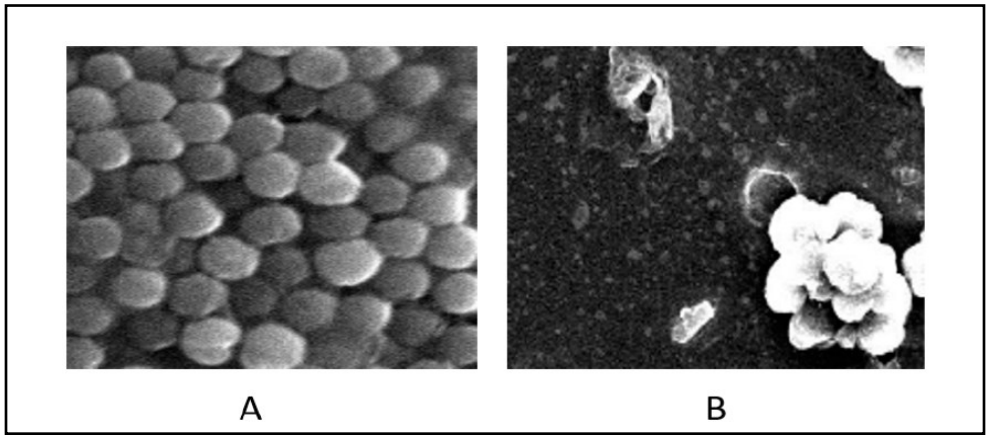

Figure 5 *Staphylococcus aureus* biofilm analyzed by Scanning Electron Microscopy (SEM) (A) Before treatment with C-10 massoialactone, (B) After treatment with C-10 massoialactone [42]

A low concentration of C-10 massoialactone successfully killed the microorganism at a short contact time. Furthermore, Valliammai *et al.* [49] demonstrated that baicalein reduced the C-reactive protein level and procalcitonin in *S. aureus* biofilm by inhibiting Agr quorum sensing system and lowering the expression of *Staphylococcal Enterotoxin* A (SEA). The combination with another phytochemical, such as linezolid, enhanced the activity of baicalein is further supported by Liu *et al.* [26], in which the combination of baicalein with linezolid had a significant inhibitory effect on the MRSA biofilm.

Antimicrobial Coating

AGXX and GOX antimicrobial coatings have also been used to control biofilm growth. AGXX is an effective antimicrobial against many Gram-positive and Gram-negative bacteria [50]. Vaishampayan *et al.* [51] suggested that AGXX can act as a biofilm inhibitor, while the combination of GOX and AGXX may be effective against MRSA. These materials may affect the ability of *S. aureus* to survive in biofilms by disrupting the transcription system crucial for the intracellular survival and the pathogenesis of MRSA. AGXX inhibited the *S. aureus* biofilm formation by 46%. Furthermore, the cellulose-based fibers have also been shown to influence the expression of siderophore genes suggesting that they impose stress on the bacterial cell and create iron-deficient conditions. GOX and AGXX, or their combination, offer numerous potential medical equipment, biocides, and agriculture applications. Apart from that, AGXX has also been demonstrated to kill *S. aureus,* as portrayed through disk diffusion assay and growth kinetics experiments. Hydrogel dressings loaded with GOX have been shown to kill *S. aureus* and promote wound healing [52 - 55].

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CONCLUSION

The high prevalence of MRSA makes it essential to discover new antibiofilm treatments. Disinfectants, bioactive glasses, antibiotics, drugs, plants, nanoparticles, bacteriophages, phytochemicals, and antimicrobial coating are the potential strategies to manage a wide range of infections mediated by *S. aureus* and MRSA biofilms. Further research is necessary to examine the molecular basis of the control of *S. aureus* and MRSA biofilms in healthcare and industrial settings.

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AUTHOR'S CONTRIBUTION

Hanis Fadhlina Hamdan and Nawal Zulkiply researched, wrote, and revised the article. Mohd Fakharul Zaman Raja Yahya conceptualized the central research idea, provided the theoretical framework, supervised the research progress, and approved the article submission.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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