



Bacterial Resistance to Disinfectants in Hospitals of the Medical City, Baghdad, Iraq

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ABSTRACT

Aims Biofilm formation by bacteria causing healthcare-associated infections poses significant challenges in healthcare and clinical environmental settings, as these biofilms contribute to the emergence of antimicrobial resistance, chronic infections, and contamination of medical equipment, complicating infection control and sanitation strategies. This study investigated the biofilm development of *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* following exposure to three disinfectant formulations at different concentrations.

Materials & Methods This experimental study was done on 40 bacterial isolates collected from various hospital surfaces and medical equipment from different hospitals in Medical City in Baghdad. The first disinfectant comprised 45% ethyl alcohol, 5% isopropyl alcohol, and dodecyl dimethyl ammonium chloride. The second formulation included quaternary ammonium propionate, chlorhexidine digluconate, and a 2% glutaraldehyde solution. The third consisted of 10% dodecyl dimethyl ammonium chloride and 2% benzalkonium chloride. Biofilm formation was assessed using the Crystal Violet assay. Data were analyzed by SPSS 20.

Findings Most bacterial strains exhibited moderate to strong biofilm formation across all treatments, with some instances of weak biofilm development noted after the application of the second disinfectant. This suggests that disinfectant efficacy against biofilm-associated bacteria remains limited.

Conclusion Most bacterial strains exhibit moderate to strong biofilm formation across all treatments.

Keywords Biofilm; Healthcare-Associated Infection; Disinfectant; Quaternary Ammonium Compounds (QACs); Ethanol

CITATION LINKS

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Introduction

In some countries, such as Greece and Cyprus, there is a lack of information on the use of disinfectants, and incorrect practices are adopted by workers in both countries regarding the amount of expired or leftover detergents. This issue poses a problem for infection control management in hospitals [1]. Laboratory studies have identified multiple mechanisms, by which bacteria can develop tolerance or resistance to quaternary ammonium compounds (QACs) and antibiotics [2].

Bacterial biofilms are complex communities of bacteria that adhere to surfaces and are held together by a self-produced polymer matrix, primarily composed of polysaccharides, secreted proteins, and extracellular DNA [3]. Biofilms tend to occur in healthcare environments and are linked to healthcare-associated infections (HAIs), making them generally less susceptible to antibacterial agents [4].

The presence of biofilms reduces the vulnerability of both Gram-positive and Gram-negative bacteria to disinfectants, with the greatest impact observed in Gram-negative bacteria [5]. Medical equipment, such as surgical instruments, cardiac prostheses, vascular and urinary catheters, implants, analytical and diagnostic tools, and endotracheal tubes, is particularly vulnerable to the growth of biofilms [6]. The persistence of biofilms on medical devices not only complicates treatment efforts but also increases the risk of severe infections, prolonging hospital stays and elevating healthcare costs [7]. Furthermore, the ineffectiveness of conventional disinfectants against biofilms has led researchers to explore novel antimicrobial agents and strategies, including the use of bacteriophages and enzymatic treatments, which may show increased efficacy against biofilm-associated bacteria [8].

Research indicates that education and training regarding appropriate disinfectant usage are essential to improving infection control practices [9]. For instance, healthcare workers must be aware of the correct concentrations and application methods of disinfectants to enhance their efficacy against biofilm-forming bacteria. Implementing routine assessments and monitoring protocols can also contribute significantly to reducing the risk of HAIs linked to biofilm development [10]. Therefore, addressing the challenges posed by biofilms in healthcare settings is crucial for improving patient safety and infection control outcomes.

Metallic devices and implants, such as stainless steel, titanium, and titanium alloys, are widely used in the medical industry. These materials interact with bodily fluids and microorganisms, which can change their microstructure, induce biocorrosion, and affect the biological properties of the patient [11]. Bacteria form biofilms in response to environmental stresses such as UV radiation, desiccation, limited nutrients,

extreme pH, extreme temperatures, high salt concentrations, high pressure, and antimicrobial agents. The events leading to bacterial biofilm formation are complex [12]. Biofilms are believed to account for over 65% of nosocomial infections, around 80% of chronic infections, and 60% of all bacterial infections in humans [13].

Due to the substantial increase in the use of disinfectants containing quaternary ammonium compounds (QACs) in healthcare and community settings during the COVID-19 pandemic, there is growing concern that heavy use might cause bacteria to develop resistance to QACs or contribute to antibiotic resistance [2]. Excessive and high-concentration use of QACs leads to environmental pollution and the generation of resistant strains [14]. Disinfectants are classified into different categories: alcohols, aldehydes, quaternary ammonium compounds, halogens, chlorhexidine, and oxidizing agents [15]. Alcohols, phenols, and quaternary ammonium compounds are widely used disinfectants with varying active ingredients and mechanisms. They operate in two stages: primary and secondary. The effectiveness of these disinfectants is influenced by their shelf life, which can be impacted by temperature, sunlight exposure, and organic matter [16]. Benzalkonium chloride (BAC) is a mixture of alkyl benzyl dimethyl ammonium chloride compounds and is the most common active ingredient in surface cleaning and disinfecting products [17].

Currently, there is a new concern regarding alcohol-tolerant bacteria [18]. A study demonstrated the inhibitory effects of certain disinfectants, such as ethanol and Dettol, on MRSA, *Acinetobacter baumannii*, *E. coli*, and *Klebsiella* species, and significantly revealed that all tested bacteria were resistant to ethanol at all concentrations [19]. This resistance can be attributed to several factors, including the overuse of antiseptics, mechanisms of resistance that may develop in bacteria through mutation and the acquisition of resistance genes from other organisms, and biofilm formation, in which bacteria like *Acinetobacter baumannii* tend to form biofilms that can protect them from the action of disinfectants [20].

Moreover, the emergence of disinfectant-resistant strains poses a significant challenge to infection control in healthcare settings. Studies have shown that inappropriate use of disinfectants can lead to selective pressure, facilitating the development of resistant bacterial strains [21]. Additionally, the ineffectiveness of traditional disinfectants against biofilm-associated bacteria can complicate treatment protocols and increase the risk of healthcare-associated infections (HAIs) [22].

The aim of this research was to evaluate the biofilm formation capabilities of four healthcare-associated bacteria when exposed to different disinfectant formulations at varying concentrations, assess the

efficacy of these disinfectants in disrupting biofilms, and highlight the resilience of biofilms under suboptimal disinfectant conditions. Understanding the biofilm formation capabilities of these bacteria is crucial because biofilms are known to significantly contribute to the persistence of infections in healthcare settings, leading to increased morbidity and healthcare costs. By investigating how different disinfectant formulations perform against biofilms, this study provides valuable insights that can inform infection control protocols and enhance patient safety. Furthermore, the findings from this research could guide the development of more effective disinfectant strategies tailored to combat biofilm-associated infections, ultimately reducing the incidence of HAIs and improving overall clinical outcomes. Given the rising concern over antibiotic resistance, this study also addresses a critical gap in current literature by exploring alternative approaches to infection prevention, emphasizing the need for rigorous evaluation of disinfectants in real-world healthcare environments.

Materials and Methods

Bacterial isolation

This experimental study used bacterial strains collected from various hospital surfaces and medical equipment, such as neonatal incubators, surgical tools, patient beds, bed corners, urinary catheters, and air conditioning openings in Medical City in Baghdad from 2023 to the end of March 2024. A total of 40 bacterial isolates (13 isolates of *Acinetobacter baumannii*, 9 isolates of *Klebsiella pneumoniae*, 13 isolates of *Pseudomonas aeruginosa*, and 5 isolates of *Burkholderia cepacia*) were identified using the VITEK 2 system.

Table 1. Disinfectant used in the study

Disinfectants	Active ingredient
First	45% Ethyl Alcohol (Ethanol), 5% Propane-2-ol (Isopropyl Alcohol), Didecyl Dimethyl Ammonium Chloride (DDAC), Perfume and Deionized Water
Second	- For the pre-disinfection step aniosome used (quaternary ammonium propionate, chlorhexidine digluconate, non-ionic surfactants, enzyme complex (protease, lipase, and amylase) perfume, dye, excipients - Disinfection step steranos used (2% glutaraldehyde solution buffered at pH 6 (sodium citrate))
Third	10% Didecyl Dimethyl Ammonium Chloride (DDAC), 2% benzalkonium chloride, anti-corrosive agent, auxiliary substances and purified water

Disinfectants

Three different disinfectants were used (Table 1). The selection was based on their documented use in sterilization protocols within the hospitals, from which the bacterial samples were sourced. This approach was undertaken to investigate the etiology

of disinfectant resistance and to identify the persistence of bacteria despite the implementation of standard sterilization procedures in these healthcare settings.

Table 2. Disinfectant concentration and percentage used in the experiment

Disinfectants	Percentage (%)	Concentration (μL)	Water (μL)	Bacteria (μL)
First	25	45	135	20
	50	90	90	20
	75	135	45	20
Second	25	45	135	20
	50	90	90	20
	75	135	45	20
Third	25	45	135	20
	50	90	90	20
	75	135	45	20

Biofilm formation

Biofilm formation was assessed using the crystal violet assay, following the methods of Hashem *et al.* [23] and Singh *et al.* [24] with some modifications. The *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* strains were activated by growing them in double-strength brain heart infusion with 0.5% glucose at 37°C for 24 hours. A sterile flat-bottom 96-well microtiter plate was inoculated with 200 μL of the diluted cultures aseptically (the differences in the three surface disinfectants were measured between isolates according to sub-MIC for each), and each isolate was added in triplicate wells. It is important to mention that the MIC of the three different disinfectants was determined at varying concentrations (Table 2). Additionally, positive and negative controls were prepared.

Table 3. Biofilm degree of *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* isolates

Mean OD (600nm)	Biofilm degree	Reference
OD≤ODc	Non-adherent	Hashem <i>et al.</i> , 2021 [23]
ODc<OD≤2*ODc	Weakly adherent	
2*ODc<OD≤4*ODc	Moderately adherent	
4*ODc<OD	Strongly adherent	

OD = Optical density; O.D.c is the O.D. of the negative control. Because the assay was performed in triplicates and some cases had outliers, the median—rather than the mean—was used for analysis

The wells were washed with saline three times and left to dry. Methanol was used to fix the adherent cells. Fixed adherent cells were stained with 1% (W/V) crystal violet for 15 minutes, and excess stain was removed by washing (Figure 1).

The plates were then left to dry. Crystal violet bound to the adherent cells was re-dissolved in glacial acetic acid. The optical density (OD) was measured at 600nm in a plate reader (Biotek, USA), and the median of three readings was taken. The strength of the biofilm was classified according to the OD readings (Table 3).

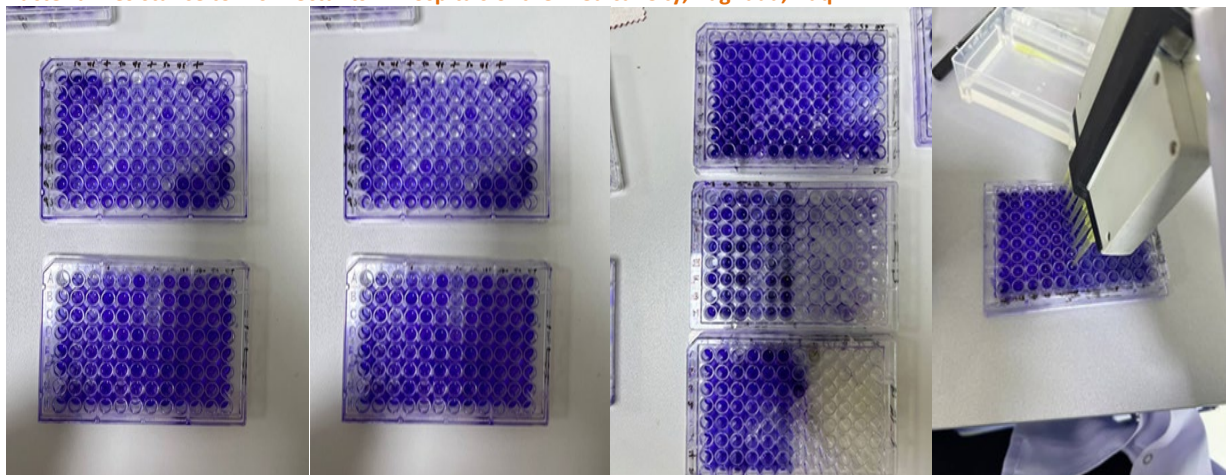


Figure 1. Microtiter plates filled with crystal violet, disinfectants, and different isolates of bacteria

Data analysis

Data were analyzed by SPSS 20 using the chi-square test to obtain significant differences between the frequency numbers collected regarding the ability of biofilm formation. The results were presented as mean $\pm$ S.E., and a significant difference was considered at $p \leq 0.05$.

Findings

Disinfectant resistance among bacteria, such as *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* in hospital environments is a growing concern [25]. These bacteria can exhibit resistance to disinfectants, like ethanol, DDAC, BAC, and quaternary ammonium propionate, even at varying concentrations.

The effect of the three disinfectants (25%, 50%, and 75%) against the biofilm of 40 Gram-negative bacteria (13 isolates of *Acinetobacter baumannii*, 9 isolates of *Klebsiella pneumoniae*, 13 isolates of *Pseudomonas aeruginosa*, and five isolates of *Burkholderia cepacia*) was tested, and the results showed that all isolates formed biofilms.

For *Acinetobacter baumannii*, biofilm formation after treatment with the first disinfectant (ethanol, DDAC) at varying concentrations revealed significant insights into the relationship between disinfectant and biofilm development, notably showing moderate and strong biofilm formation at 25% and 50%

concentrations, respectively. All isolates exhibited strong biofilm formation at the highest concentration of 75%.

After treatment with the second disinfectant (quaternary ammonium propionate, chlorhexidine digluconate), there was a significant difference between treatments at 25% and 75% concentrations. Most isolates formed strong biofilms, while fewer formed moderate biofilms, and one isolate formed a weak biofilm at 25%. Additionally, two isolates formed weak biofilms at 75%.

For the third disinfectant (DDAC, benzalkonium chloride), most isolates formed strong biofilms, and one isolate formed a moderate biofilm, indicating a significant difference (Table 4).

When the nine *Klebsiella pneumoniae* isolates were treated with the mentioned disinfectants, the first sterilizer (ethanol + DDAC) resulted in six isolates forming strong biofilms and three forming moderate biofilms at concentrations of 25% and 50%. In contrast, all the isolates formed strong biofilms when exposed to the first disinfectant at a concentration of 75% and to all concentrations of the third disinfectant (DDAC, BAC). Following treatment with the second disinfectant (quaternary ammonium propionate), the 25% and 50% concentrations induced moderate to strong biofilm formation, whereas the 75% concentration resulted in weak, moderate, and strong biofilm formation across the isolates (Table 5).

Table 4. Biofilm formation of *Acinetobacter baumannii* after the treatment with disinfectants

Isolate (n=13)	Type of sterilizer	Concentration	Ability of biofilm formation			p-value
			Weak	Moderate	Strong	
<i>Acinetobacter baumannii</i>	Sterilizer 1	1	0(0)	6(46.15)	7(53.85)	0.7
		2	0(0)	8(61.54)	5(38.46)	0.4
		3	0(0)	0(0)	13(100)	-
	Sterilizer 2	1	1(7.69)	3(23.08)	9(69.23)	0.02
		2	0(0)	5(38.46)	8(61.54)	0.4
		3	2(15.38)	3(23.08)	8(61.54)	0.05
	Sterilizer 3	1	0(0)	1(7.69)	12(92.31)	0.002
		2	0(0)	0(0)	13(100)	-
		3	0(0)	1(7.69)	12(92.31)	0.002

Table 5. Biofilm formation of *Klebsiella pneumoniae* after the treatment with disinfectants

Isolate (n=9)	Type of sterilizer	Concentration	Ability of biofilm formation			p-value
			Weak	Moderate	Strong	
<i>Klebsiella pneumoniae</i>	Sterilizer 1	1	0(0)	3(33.33)	6(66.67)	0.05
		2	0(0)	3(33.33)	6(66.67)	0.05
		3	0(0)	0(0)	9(100)	-
	Sterilizer 2	1	0(0)	5(55.56)	4(44.44)	0.7
		2	0(0)	5(55.56)	4(44.44)	0.7
		3	3(33.33)	3(33.33)	3(33.33)	1.0
	Sterilizer 3	1	0(0)	0(0)	9(100)	-
		2	0(0)	0(0)	9(100)	-
		3	0(0)	0(0)	9(100)	-

Table 6. Biofilm formation of *Pseudomonas aeruginosa* after the treatment with disinfectants

Isolate (n=13)	Type of sterilizer	Concentration	Ability of biofilm formation			p-value
			Weak	Moderate	Strong	
<i>Pseudomonas aeruginosa</i>	Sterilizer 1	1	0(0)	9(69.23)	4(30.77)	0.1
		2	0(0)	7(53.85)	6(46.15)	0.8
		3	0(0)	5(38.46)	8(61.54)	0.3
	Sterilizer 2	1	1(7.69)	6(46.15)	6(46.15)	0.1
		2	2(15.38)	7(53.85)	4(30.77)	0.2
		3	5(38.46)	2(15.38)	6(46.15)	0.36
	Sterilizer 3	1	0(0)	3(23.08)	10(76.92)	0.05
		2	0(0)	1(7.69)	12(92.31)	0.002
		3	1(7.69)	6(46.15)	6(46.15)	0.1

Table 7. Biofilm formation of *Burkholderia cepacia* after the treatment with disinfectants

Isolate (n=5)	Type of sterilizer	Concentration	Ability of biofilm formation			p-value
			Weak	Moderate	Strong	
<i>Burkholderia cepacia</i>	Sterilizer 1	1	0(0)	3(60)	2(40)	0.7
		2	0(0)	3(60)	2(40)	0.7
		3	0(0)	2(40)	3(60)	0.7
	Sterilizer 2	1	0(0)	0(0)	5(100)	-
		2	0(0)	0(0)	5(100)	-
		3	0(0)	0(0)	5(100)	-
	Sterilizer 3	1	0(0)	0(0)	5(100)	-
		2	0(0)	0(0)	5(100)	-
		3	0(0)	0(0)	5(100)	-

When exposed to disinfectant 1 (ethanol + DDAC) at varying concentrations, the 13 *Pseudomonas* isolates exhibited moderate to strong biofilm formation. With the second sterilizer (quaternary ammonium propionate), the isolates formed moderate to strong biofilms; however, numerous isolates formed weak biofilms at the 25% and 50% concentrations of the disinfectant. At the 75% concentration, five isolates formed weak biofilms, while six formed strong biofilms. The majority of the 13 isolates formed strong biofilms, with several forming moderate biofilms at the 25% and 50% concentrations of the third disinfectant (DDAC, BAC). At the 75% concentration, one isolate formed a weak biofilm, while the remaining isolates exhibited moderate to strong biofilms (Table 6).

In *Burkholderia cepacia*, all five isolates formed strong biofilms after treatment with the second disinfectant (quaternary ammonium propionate) and the third disinfectant (DDAC, BAC). Regardless of the concentration used, treatment with the first disinfectant (ethanol + DDAC) resulted in moderate to strong biofilm formation (Table 7).

Discussion

Several studies have reported that in high, middle, and low-income healthcare settings, antiseptics, low-

level disinfectants, and hand hygiene products can be susceptible to bacterial contamination. Numerous studies assessing the susceptibility of *A. baumannii* biofilms to disinfectants like benzalkonium chloride and chlorhexidine have shown the ability of both hospital and environmental isolates to form biofilms on surfaces, such as glass and ceramics [26].

Non-fermentative gram-negative bacteria are the predominant contaminants, with *Burkholderia cepacia*, *Pseudomonas aeruginosa*, and *Achromobacter* spp. being the most frequently recognized. These organisms are particularly concerning due to their inherent resistance to multiple antibiotics, which complicates treatment options and increases the risk of severe infections in vulnerable patient populations, such as those in intensive care units. Enterobacterales are also notable contaminants, with *Serratia* spp. being the most common; these bacteria can lead to a range of healthcare-associated infections, including bloodstream infections and pneumonia. The presence of these pathogens underscores the urgent need for effective infection control measures, as their ability to form biofilms further enhances their persistence in clinical settings and contributes to outbreaks. Moreover, understanding the prevalence and resistance patterns of these contaminants is

critical for developing targeted interventions and improving patient safety in healthcare environments [27-29].

In this research, all the isolates were capable of forming biofilms, and most of them had the ability to form strong and moderate biofilms. This finding is consistent with the studies by Mougin et al. [30] and Stempel et al. [31], which demonstrated that the overuse and misuse of disinfectants such as BAC lead to the persistence of opportunistic bacteria like *Pseudomonas* and stimulate biofilm formation.

This work provides insights into some bacterial adaptation mechanisms and stress responses when exposed to sub-lethal disinfectants. Biofilm formation can be induced by exposure to disinfectants, serving as a protective barrier against antimicrobial agents and leading to increased exopolysaccharide (EPS) production, accelerated cell proliferation, and enhanced adhesion. Bacteria within biofilms are often in a metabolically inactive state, making them less susceptible to both disinfectants and antibiotics [32, 33]. Prior research has indicated that these biofilms exhibit distinct shifts in EPS composition and quantity in response to subinhibitory levels of various disinfectants [34]. Biofilms also facilitate horizontal gene transfer among bacteria, further spreading resistance traits. Additionally, changes in the bacterial cell envelope through various mechanisms, such as increased surface hydrophobicity or changes in surface charge, can reduce permeability to disinfectants, preventing these agents from reaching their targets within the cell. This intrinsic resistance is particularly notable in gram-negative bacteria [35].

Some studies have demonstrated that certain bacterial strains, such as *Burkholderia cepacia*, exhibit natural resistance to antibiotics, antiseptics, and disinfectants, particularly quaternary ammonium compounds and chlorhexidine, along with biofilm formation [36, 37].

An investigation of surface swab samples from intensive care units (ICUs) revealed that 95.8% of *Acinetobacter baumannii* isolates produced biofilms; among these, 45.83% exhibited strong biofilm formation, 29.16% showed moderate biofilm formation, and 20.83% demonstrated weak ability for biofilm formation. The capacity of these bacterial isolates, particularly those from bed rails, to form biofilms presents a significant infection risk for hospitalized patients, healthcare workers, and visitors. This is due to the persistent nature of these biofilms, which can harbor and transmit pathogenic microorganisms in hospital environments, thereby complicating infection control measures and increasing the potential for nosocomial infections [3, 38]. Furthermore, the ability of biofilms to resist both antimicrobial agents and host immune responses makes them particularly challenging to eradicate, often leading to prolonged hospital stays and increased healthcare costs. The high prevalence of

biofilm-producing *Acinetobacter baumannii* underscores the necessity for rigorous surface decontamination protocols and routine monitoring to mitigate the risk of infection transmission. Additionally, understanding the biofilm formation dynamics of these pathogens can inform the development of novel therapeutic strategies aimed at disrupting biofilms and enhancing patient safety in critical care settings.

Commonly used disinfectants often exhibit significant limitations when it comes to effectively targeting biofilm-associated bacteria, which presents substantial challenges in healthcare and clinical environmental settings. One major limitation is that biofilms create a protective matrix that shields embedded bacteria from the full effects of disinfectants, significantly reducing their efficacy. Additionally, many disinfectants can be inactivated by organic matter, such as blood and tissue, which frequently accumulate on surfaces in healthcare environments, further compromising their effectiveness. Certain bacterial species, like *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, can also develop resistance mechanisms that allow them to survive even in the presence of potent disinfectants. The variability in disinfectant effectiveness against different bacterial strains can lead to inconsistent outcomes, allowing resistant populations to thrive and potentially cause HAIs. Furthermore, the sporadic application and improper dilution of disinfectants can lead to suboptimal conditions for microbial control, resulting in persistent biofilm formation. This situation highlights the urgent necessity for healthcare facilities to implement more comprehensive infection prevention strategies specifically targeting biofilms. Innovative approaches, such as the development of novel antimicrobial agents or the use of combination therapies, should be prioritized to enhance disinfectant effectiveness. Future studies must address the mechanisms of biofilm resilience and the interactions between disinfectants and biofilms to identify more effective interventions. By focusing on these critical areas, researchers can help improve infection control practices, ultimately reducing the incidence of HAIs and safeguarding patient health in clinical settings.

Conclusion

Most bacterial strains exhibit moderate to strong biofilm formation across all treatments.

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