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Biofilms: Mechanisms of Formation and Strategies for Control in Clinical Settings

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In Loving Memory of Late Professor Doctor ""Mohamed Refaat Hussein Mahran""

Abstract

Background: Biofilms are structured communities of bacteria that adhere to surfaces and are embedded in an extracellular polymeric substance (EPS) matrix. These biofilms are a major concern in medical settings due to their resistance to antibiotics and role in chronic infections.

Aim: This study aims to explore the mechanisms behind biofilm resistance and the emerging strategies to combat biofilm-associated infections.

Methods: A comprehensive review of current literature was conducted, focusing on the structural and functional aspects of biofilms, including nutrient limitation, stress responses, and the role of persister cells. The review also examined new approaches to prevent and disrupt biofilm formation.

Results: The findings indicate that biofilm resistance is multifaceted, involving reduced metabolic activity, the protective role of the EPS matrix, and adaptive responses to stress. Emerging strategies, such as the use of antimicrobial peptides, biosurfactants, and anti-biofilm coatings, show promise in enhancing the efficacy of treatments against biofilm-associated infections.

Conclusion: Biofilm-related infections pose significant challenges due to their complex resistance mechanisms. Novel approaches targeting biofilm formation and persistence are crucial for improving treatment outcomes and preventing chronic infections.

Keywords: Biofilm, antibiotic resistance, persister cells, extracellular polymeric substance, antimicrobial peptides, quorum sensing, biofilm disruption strategies.

Introduction:

Biofilms are complex, structured communities of microorganisms embedded in a self-produced extracellular matrix (ECM) composed of extracellular polymeric substances (EPS), including polysaccharides, proteins, lipids, nucleic acids, and other components [1]. These communities are sessile, meaning that they adhere to both biological and non-biological surfaces, and are prevalent in a variety of environments, ranging from natural ecosystems to industrial settings and the human body [2,3]. Biofilms are of particular concern in medical contexts, as they are responsible for more than 80% of microbial infections in the human body, making them a significant focus of research and clinical practice [4]. The formation of biofilms on medical devices and tissues poses a major challenge for infection control due to their inherent resistance to antimicrobial agents and the host immune system. The development of biofilms begins when planktonic, or free-floating, bacteria adhere to a surface and begin to secrete EPS, which helps them to stick together and to the surface [5]. This initial adhesion is followed by the production of more EPS, leading to the formation of microcolonies and the development of a mature biofilm with a complex three-dimensional structure [6]. These biofilms can be found on a wide range of surfaces, including medical devices such as catheters, prosthetic heart valves, and contact lenses, as well as on tissues within the body, such as the lungs of cystic fibrosis patients [7,8]. The ECM of a biofilm serves multiple functions, including providing structural support, retaining water, protecting the bacteria from

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environmental stresses, and facilitating the exchange of nutrients and waste products [9]. The ability of biofilms to protect the bacteria within them from antibiotics and the host immune system makes them particularly difficult to treat and eradicate [10].

Biofilms are not only a problem in medical settings but also pose significant challenges in industrial and environmental contexts. In industrial settings, biofilms can cause the clogging of filters, corrosion of pipes, and contamination of products [11]. In natural environments, biofilms play an important role in nutrient cycling and the degradation of organic matter, but they can also be harmful, such as when they form on the surfaces of ships, leading to increased drag and fuel consumption [12]. The persistence and resistance of biofilms in various environments have led to increased interest in understanding their formation, structure, and the mechanisms by which they resist antimicrobial agents [13]. The formation of biofilms is a complex process that involves several stages. The initial stage involves the transport and adhesion of planktonic bacteria to a surface, which is often facilitated by the adsorption of suspended particles and organic species from the surrounding fluid [14]. Once attached, the bacteria begin to produce EPS, which helps them to adhere more firmly to the surface and to each other, leading to the formation of microcolonies [15]. As the biofilm matures, it develops a three-dimensional structure with channels that allow for the circulation of nutrients and waste products [16]. The final stage of biofilm development involves the dispersal of bacterial cells from the biofilm, which can then colonize new surfaces and form new biofilms [17]. This ability to disperse and form new biofilms is one of the reasons why biofilm-associated infections are so difficult to eradicate [18].

One of the most significant challenges in treating biofilm-associated infections is the inherent resistance of biofilms to antimicrobial agents. This resistance is due to several factors, including the physical barrier provided by the ECM, the slow growth rate of bacteria within the biofilm, and the presence of dormant cells that are less susceptible to antibiotics [19]. The ECM can slow the diffusion of antibiotics into the biofilm, making it difficult for them to reach and kill the bacteria within [20]. Additionally, the slow growth rate of bacteria within the biofilm means that they are less likely to be affected by antibiotics that target actively dividing cells [21]. Finally, the presence of dormant cells, also known as persister cells, within the biofilm can lead to the survival of a small population of bacteria even after treatment with high concentrations of antibiotics, leading to the recurrence of the infection [22]. The resistance of biofilms to antimicrobial agents is a significant concern in medical settings, where biofilm-associated infections can lead to chronic infections, increased morbidity and mortality, and higher healthcare costs [23]. These infections are particularly problematic in patients with implanted medical devices, such as catheters, prosthetic heart valves, and joint prostheses, where biofilms can form on the surface of the device and lead to persistent infections that are difficult to treat [24]. In some cases, the only way to treat a biofilm-associated infection is to remove the infected device, which can be invasive and carry significant risks for the patient [25].

In addition to their resistance to antibiotics, biofilms also protect the bacteria within them from the host immune system. The ECM can act as a physical barrier that prevents immune cells, such as phagocytes, from reaching and killing the bacteria within the biofilm [26]. Additionally, the bacteria within the biofilm can produce enzymes that degrade components of the immune system, such as complement proteins, further protecting them from immune attack [27]. This immune evasion is one of the reasons why biofilm-associated infections can become chronic and difficult to eradicate [28]. The impact of biofilms on human health and industrial processes has led to a significant amount of research into strategies for preventing and treating biofilm-associated infections. One approach is to develop new antimicrobial agents that are more effective at penetrating the biofilm and killing the bacteria within [29]. Another approach is to prevent the formation of biofilms in the first place, by coating surfaces with materials that prevent bacterial adhesion or by disrupting the signaling pathways that bacteria use to coordinate biofilm formation [30]. There is also interest in developing strategies to disrupt existing biofilms, such as by using enzymes that degrade the ECM or by using mechanical methods to remove the biofilm from the surface [31].

Despite the challenges posed by biofilms, there is hope that new technologies and approaches will lead to more effective strategies for preventing and treating biofilm-associated infections. Advances in our understanding of the molecular mechanisms underlying biofilm formation and resistance have led to the identification of new targets for antimicrobial therapy, and there is ongoing research into the development of new drugs and materials that can prevent or disrupt biofilms [32]. Additionally, there is interest in using alternative approaches, such as bacteriophage therapy, which uses viruses that specifically target and kill bacteria, as a way to treat biofilm-associated infections [33]. In conclusion, biofilms are complex communities of microorganisms that are responsible for a significant proportion of infections in both medical and industrial settings. Their resistance to antimicrobial agents and the host immune system makes them particularly difficult to treat and eradicate. However, advances in our understanding of biofilm biology

and the development of new strategies for preventing and treating biofilm-associated infections offer hope for more effective management of these challenging infections in the future. Further research is needed to continue to develop new approaches for combating biofilms and to improve outcomes for patients affected by biofilm-associated infections.

Biofilm Formation and Adhesion Mechanisms:

The formation and development of biofilms involve five distinct stages, starting with the surface adhesion of microbial cells, followed by the growth and maturation of the biofilm (**Figure 1**). This process is influenced by various factors, including sedimentation, Van der Waals forces, hydrodynamic forces, Brownian motion, and electrostatic or hydrophobic interactions, which play a crucial role in bacterial deposition [34]. Specific surface-linked proteins, such as protein A [35], SasG [36,37], fibronectin-binding protein [38], biofilm-associated protein (BAP) [39,40], and OmpA, are instrumental in the initial stages of biofilm formation. Some microbial species may not adhere directly to surfaces but can attach to existing cells or matrices. Ultimately, microbial cells within biofilms are encased in an extracellular matrix composed of various biomolecules, including nucleic acids, proteins, lipids, and polysaccharides [41]. The formation and maturation of biofilms are also influenced by quorum sensing (QS), a cell-to-cell communication mechanism mediated by small signaling molecules [42]. The extracellular matrix of biofilms provides protection to bacterial cells against external stress conditions, though it does not necessarily act as a physical barrier to antimicrobials. Biofilm dispersion can be triggered either chemically or through mechanical stress. Anderl et al. demonstrated that ampicillin could penetrate the β-lactamase-deficient biofilm of Klebsiella pneumoniae, while it was unable to infiltrate the biofilm of the β-lactamase-producing wild-type strain. In the latter case, the ampicillin was degraded before it could penetrate the biofilm [43].

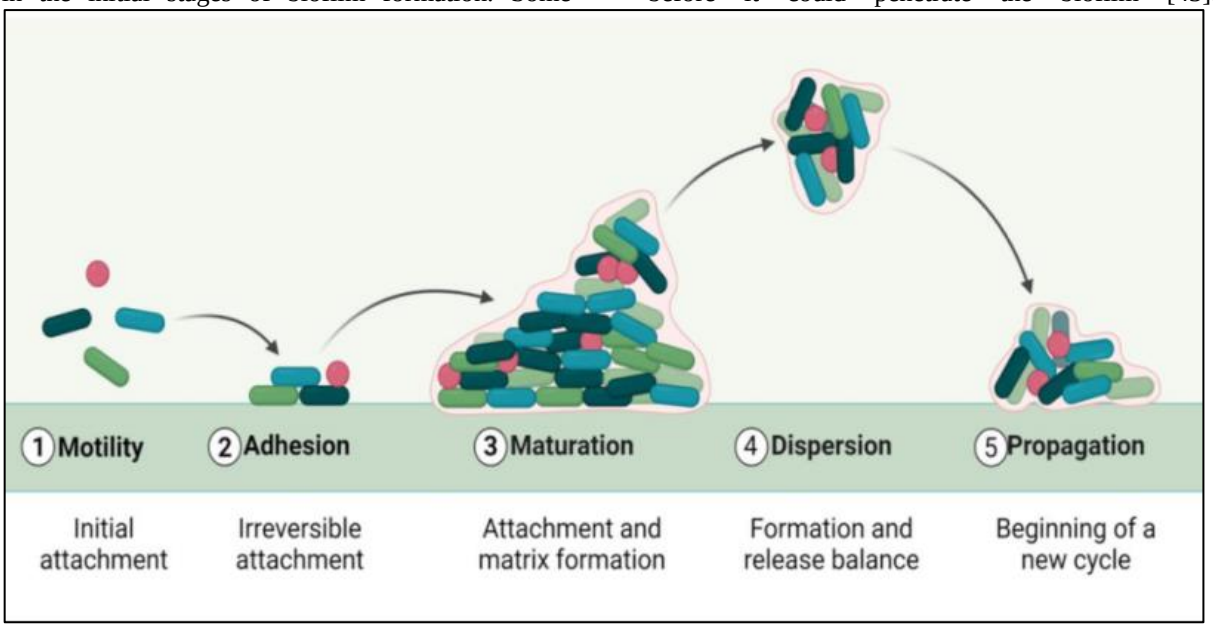

Figure 1: Biofilm Formation and Adhesion Mechanism

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Biofilm formation on surfaces generally occurs in three main stages. Initially, cells attach to a surface, then assemble into microcolonies, and eventually differentiate into a mature biofilm structure. Following the complete development of a biofilm, its disassembly or dispersion occurs through both mechanical and active processes [39]. Bacterial deposition is primarily mediated by sedimentation, Brownian motion, and hydrodynamic forces, while adhesion to the substrate is governed by Lifshitz–Van der Waals, acid-base, hydrophobic, and electrostatic interaction forces [40]. Surface-associated proteins, including OmpA, fibronectin-binding proteins [31], protein A [32], SasG [43,44], biofilm-associated protein (BAP) [45,46], among others, play critical roles during the initial attachment stages of biofilm formation. Some species may not attach directly to surfaces but can anchor themselves to the matrix or previously formed colonies. Colonization is mediated by small signaling molecules through cell-cell communication systems, commonly referred to as quorum sensing [47], with biofilm formation being a major phenotype controlled by quorum sensing [48]. Within biofilms, bacterial cells are encapsulated in an extracellular matrix, a complex mixture of biomolecules, including proteins, polysaccharides, nucleic acids, and lipids [49]. The matrix provides protection from various stress conditions, such as exposure to antimicrobials or immune cells, although it does not act as a mechanical barrier to antimicrobial agents [50]. This was evidenced by studies showing that ampicillin could penetrate the biofilm formed by a β-lactamase-deficient strain of K. pneumoniae, whereas in the wild-type strain

possessing β-lactamase, ampicillin could not penetrate the biofilm [50], suggesting rapid degradation of ampicillin by β-lactamase before it could infiltrate the wild-type biofilm. Once bacteria begin secreting extracellular polysaccharide substances (EPS), the second stage of biofilm development, which is irreversible, commences. The secretion of EPS continues through the third stage, ensuring the secure attachment of bacteria to the surface within a thick, complex biomolecular layer [51]. The fully matured biofilm adopts a tower-like, three-dimensional structure. These towers contain small channels for the transport of nutrients, water, and waste, with cavities providing shelter for planktonic bacteria. Studies have shown that the organization and architecture of biofilms vary significantly among different bacteria, though the reasons for this variation remain unclear. The adhesive protein LapA governs biofilm formation in *P. putida* [52-54], while exopolysaccharides Pel and Psl govern biofilm formation in other pseudomonads, including *P. aeruginosa* [55-57]. Differences in extracellular matrix (ECM) components may contribute to the structural variations in biofilms. Finally, these towers either erode in small parts or slough off in large parts, leading to the release of non-surface-attached bacteria and subsequent release of fresh bacteria into the environment [58,59]. Recent studies on various bacterial species, including *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Yersinia pestis*, *Escherichia coli*, *Vibrio cholerae*, *Burkholderia cenocepacia*, *Salmonella enterica*, *Clostridium difficile*, *Klebsiella pneumoniae*, *Vibrio cholerae*, and *Bacillus subtilis*, demonstrate that an increase in c-di-GMP levels, an intracellular secondary messenger, indicates the initiation of biofilm formation and virulence [52,53,60-68,69-73]. C-di-GMP was first described as a novel secondary messenger in the allosteric activation of cellulose synthase in *Gluconacetobacter xylinus* [65]. Various c-di-GMP diguanylate cyclases and phosphodiesterases synthesized by bacteria participate in different c-di-GMP circuits [64]. C-di-GMP functions by binding to a wide range of receptors, including enzymes, adaptor proteins, transcription factors, and riboswitches [71]. Additionally, environmental cues and transducer mechanisms leading to increased c-di-GMP levels in cells have been reported. This not only promotes the production of adhesins but also aids in the secretion of the extracellular matrix [75,76]. In *P. aeruginosa*, c-di-GMP levels positively regulate the production of extracellular matrix components, such as CdrA adhesin, alginate exopolysaccharide, Pel, and Psl [63,77]. Along with c-di-GMP, small regulatory RNAs (sRNA) also regulate biofilm formation in several bacterial species [78].

Some bacterial strains can form planktonic aggregates depending on growth conditions. Previous studies suggest that some strains of *S. aureus* form large aggregates, with the formation process starting in the early exponential growth phase. A cluster of about 20 cells forms a structured population when cell density is low, but at higher densities, these structures form aggregates up to 1000μm in diameter. Extracellular polysaccharide intracellular adhesin (referred to as polymers of β 1–6 N-acetylglucosamine or PNAG after the determination of its chemical structure) [79] and spa-encoding Protein A have been reported to be responsible for extensive aggregation [3]. Studies by Alhede et al. (2011) suggested that the matrix of aggregates of *P. aeruginosa* comprises DNA and mannose-rich extracellular polysaccharide like Psl [1]. Microbial adhesion and biofilm formation are major concerns in controlling biofilm-associated infections rather than biological surface colonization. Bacteria quickly adapt to extracellular conditions, forming communities, including biofilms, to survive in diverse environmental conditions. Adhered microorganisms, those embedded in biofilms, or those hiding in cracks or crevices may evade cleaning and disinfecting procedures, leading to recontamination of food products during processing. Therefore, a significant aspect of the pre-requisite program (Good Hygienic Practices Program) of a food manufacturing plant is to ensure that microbial biofilms do not form or are effectively removed [19].

In vivo, microorganisms in their native physiological state demonstrate that surface contamination follows a successive chain, including initial microbial adhesion, strengthening the binding of the attached microorganisms through exopolymer production, growth of attached microorganisms, continued secretion of exopolymers, and localized detachment of biofilm organisms caused by occasionally high fluid shear or other detachment forces, allowing colonization of nearby surfaces [20]. Adhered and biofilm-forming microorganisms may also have other adverse effects on the colonized surface, such as decreasing heat transfer [21,22] or causing corrosion [23]. The attachment mechanism to surfaces follows an organized sequence starting with the deposition of specific adhesive proteins, which bind to the surface reversibly. Successive cell deposition creates a strong binding through cell-to-cell cohesion and cell-binding proteins. Cell adhesion molecules involved in the process are first hydrolyzed by extracellular enzymes. Bacterial adhesion is directly related to protein adsorption [24].

Bacterial Adhesion to Surfaces: The Influence of Surface Roughness

Since the report in 1940 by Heukelekian H. et al., it has been recognized that surface

characteristics significantly influence bacterial adhesion and development [80]. This remains a central research area for controlling bacterial biofilm-related diseases. Bacterial adhesion to surfaces depends on various microbiological, physical, chemical, and material-related parameters, with surface topography being widely discussed as a factor influencing bacterial adhesion [81]. Bacteria embedded within biofilms are resistant to both immunological and non-specific defense mechanisms of the body. Contact with a solid surface induces the expression of bacterial enzymes, catalyzing the formation of exopolysaccharides that promote colonization and protection. Therefore, modifying surfaces to reduce attachment can limit microorganism adhesion, such as electropolishing stainless steel. Several parameters or measures have been used to characterize material surfaces based on two-dimensional characteristics, such as the Ra (roughness average), Rt (maximum peak to valley height in the sample length), and Rz values (average maximum profile height) [82]. Among the most widely used is the surface roughness parameter (Ra), representing the arithmetic mean deviation from the average surface profile. Ra is commonly expressed in micrometers (μm), but nanometer values are sometimes reported [83].

The impact of surface roughness on biofilm formation is critical. Research suggests that surface roughness in the range of 0.2 μm Ra is pivotal for cell attachment, below which there is reduced bacterial adhesion. However, surface roughness and other surface characteristics (such as hydrophobicity, chemistry, charge, or energy) interact in complex ways to influence bacterial adhesion and biofilm formation [83]. High surface roughness can create niches that protect bacteria from shear forces, thus enhancing biofilm formation. Conversely, smoother surfaces may be less conducive to bacterial colonization, though this is not universally applicable. Surface modification strategies, including material selection and surface treatment, are key in controlling bacterial adhesion and biofilm formation in various environments.

Biofilm Models and Microstructure

The study of various biofilm model systems has significantly advanced our understanding of biofilm biology. Both in vivo and in vitro model systems are utilized to investigate biofilms. In vitro biofilm model systems are broadly categorized into three main types: closed or static models, open or dynamic models, and microcosms. Among the most commonly employed closed model systems are microtiter plate-based models, which utilize static and batch growth conditions (84). In these systems, there is no exchange of media, products, or waste materials with the external environment, leading to gradual changes in experimental conditions within the wells, such as the accumulation of signaling molecules, an increase in bacterial population, and nutrient depletion in the media. Due to their cost-effectiveness and minimal reagent requirements, microtiter plate-based models allow for multiple tests to be conducted simultaneously (85). These models are also capable of distinguishing between biofilm-deficient mutants and biofilm-forming wild-type strains (86, 87), assessing the antimicrobial and anti-biofilm properties of various compounds, and identifying factors involved in biofilm initiation, such as adhesins, pili, flagella, enzymes linked to cyclic-di-GMP metabolism, and genes responsible for extracellular polysaccharide production (88, 89).

Among the open and dynamic models, the flow displacement biofilm model is widely used to study biofilms. Unlike the microtiter plate method, this system allows for the addition of nutrients and the removal of waste products (84, 90). The dynamic biofilm formation model using perfused silicone tubes is particularly significant as it closely replicates in vivo conditions. Biofilms are formed under dynamic conditions in a silicone tube system, which is then sectioned into small pieces for further analysis (91). Microcosms represent another type of in vitro model system that closely mimics in situ conditions under controlled environments, making them suitable for studying biofilms in specific contexts such as wound, oral, stream, and dental biofilms (92-94). Both in vitro and in vivo systems can be transformed into microcosms by using the same medium and creating an artificial environment to examine cell metabolism and behavior. Additionally, there exists an ex vivo model system that involves using tissues and organs extracted from organisms for further analysis and experimentation in an artificial environment. This model is valuable for monitoring bacterial colonization and progression in specific tissues or organs. To corroborate the simplified findings from in vitro model studies, in vivo model systems should also be employed. Studying mammalian models that closely resemble humans is essential to addressing various therapeutic and diagnostic challenges. These tissue-associated model systems are primarily used to investigate lung infections, urinary tract infections, and wound infections (95). Other models, such as central venous catheter models, subcutaneous foreign body infection models, intra-peritoneal foreign body infection models, urinary tract infection models, ear, nose, and throat infection models, respiratory tract infection models, and osteomyelitis infection models, have been utilized to study these infections (88). The use of mammalian models presents certain challenges, prompting researchers to explore non-mammalian model systems such as *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Danio rerio* (96). The advantages of these models include their short generation times, lower costs, and small sizes, which facilitate maintenance in microtiter plates and enable high-throughput screening of biofilm formation.

Biofilm Ultrastructure

For the first decade following the recognition of biofilm significance and ubiquity (1978-1990), biofilms were thought to be unstructured accumulations of bacterial cells encased in exopolysaccharide matrices. This misconception arose due to flawed observational techniques. Electron microscopy, which required complete dehydration of the highly hydrated biofilm matrices, and light microscopy, which suffered from out-of-focus distortions, contributed to this early misunderstanding. Although Confocal Laser Scanning Microscopy (CLSM) was invented in the 1950s, it was not initially applied to bacterial studies because the field was focused on the planktonic phenotype. CLSM allows for optical sectioning of complex structures, eliminating out-of-focus effects, and requires no sample preparation, enabling the observation of living organisms if fluorescence is introduced to visualize the cells. The first examination of living biofilms using CLSM led to a series of revelations that form the foundation of modern biofilm concepts.

One of the most critical observations was that mature biofilms are not structurally homogeneous monolayers of microbial cells on a surface. Instead, they are heterogeneous in both time and space (97). The fundamental structural unit of the biofilm is the microcolony, and understanding basic biofilm processes such as quorum sensing, antimicrobial resistance, and detachment may depend on the physiological interactions within microcolonies in a developed biofilm. **Figure 2** depicts a mixed-species biofilm grown on a metal surface in a laboratory potable-water reactor system, highlighting both the heterogeneous nature and the presence of individual microcolonies within the biofilm. Living, fully hydrated biofilms are composed of cells (6-15% by volume) and matrix material (68-85% by volume), with the cells situated in matrix-enclosed "towers" and "mushrooms". Open water channels are interspersed between the microcolonies containing the sessile cells (98), and physical techniques have demonstrated that bulk water enters these channels, producing convective flow (99).

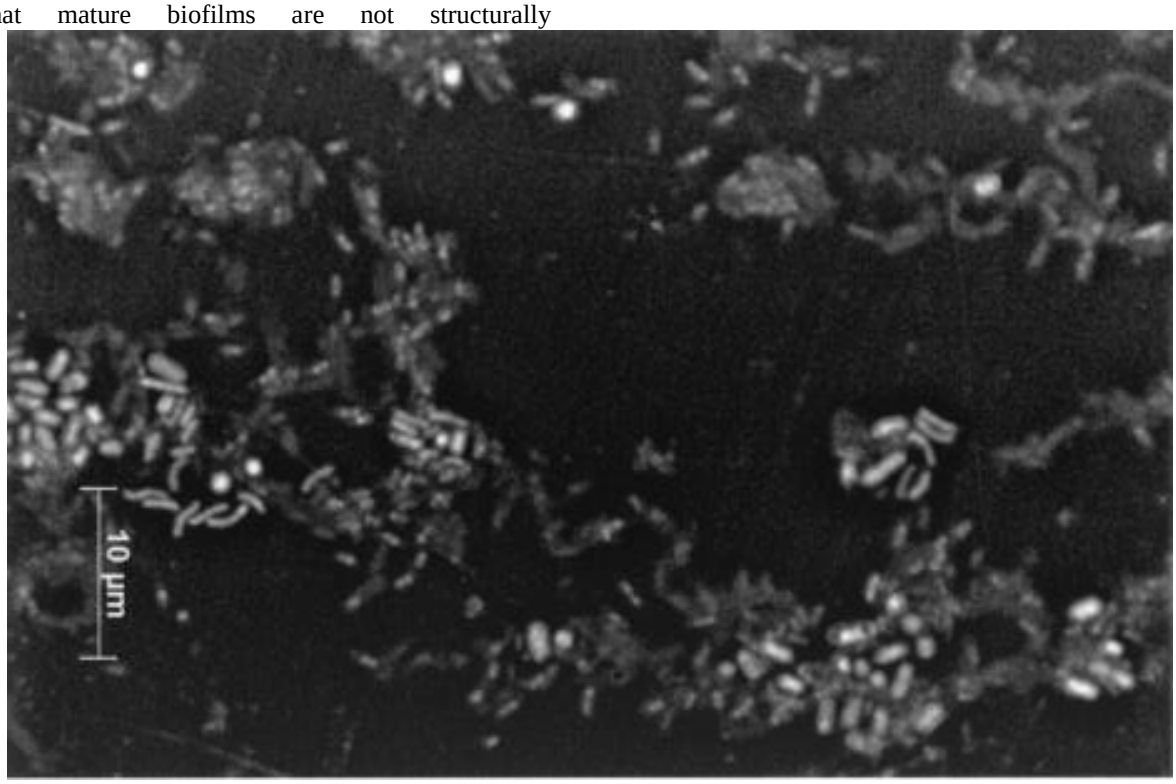

Figure 2: A mixed-species biofilm grown on a metal surface in a laboratory potable-water reactor system, highlighting both the heterogeneous nature and the presence of individual microcolonies within the biofilm.

CLSM observations of living biofilms, ranging from single-species laboratory biofilms to complex multispecies communities in natural ecosystems, have revealed that this basic community structure is universal, with minor variations. It is challenging to convey the dynamic aspects of biofilms, which are crucial, through printed work and two-dimensional figures. However, biofilms can be envisioned as a forest of rubbery towers, each attached to the colonized surface. Direct examination of biofilms in high-shear environments (100) has shown that each microcolony deforms into a tadpole shape that oscillates in the bulk fluid due to these forces. The structural feature of biofilms with the greatest impact on the outcome of chronic bacterial

infections, such as native valve endocarditis, is the propensity for individual microcolonies to break off and detach when their tensile strength is exceeded. This detachment of preformed microcolonies containing sessile cells in the antibiotic-resistant biofilm phenotype poses a significant risk of infective emboli in the first capillary bed encountered. The shedding of microcolonies from preformed biofilms on heart valves can result in stroke or severe pulmonary sequelae, and the clinical community is well aware of these consequences.

Biofilm Resistance to Antimicrobials:

Numerous mechanisms have been proposed and examined to account for the extraordinary resistance of bacteria residing within biofilms to both antibiotic treatment and phagocytosis, as illustrated in **Figure 3**. Bacteria exhibit stratified metabolic activities within biofilms due to nutrient and oxygen concentration gradients, which result in the deeper cells of the biofilm becoming less accessible to these essential resources . Since many antibiotics target actively proliferating bacteria, the less active bacteria within the biofilm exhibit inherent resistance to these treatments. Additionally, nutrient limitation activates bacterial stress responses, leading to altered gene expression and increased antibiotic tolerance . The extracellular polymeric substance (EPS) matrix of biofilms may function as a protective barrier, diffusion impediment, and reservoir of enzymes capable of degrading antibiotics. Extracellular DNA (eDNA) within the matrix may further contribute to resistance by triggering certain cellular systems . The high density and close proximity of cells in biofilms activate quorum sensing (QS) mechanisms, enabling bacteria to detect and respond to cell density changes through gene regulation. QS influences biofilm development and regulates the production of virulence factors such as enzymes and toxins, which are vital for resisting phagocytosis . Biofilms also exhibit increased rates of mutation and horizontal gene transfer, largely due to high cell density and oxidative stress . The presence of "persister cells," which can survive antibiotic treatments, further contributes to biofilms' resilience. Additional species-specific and antibiotic-specific mechanisms have been explored [100-110].

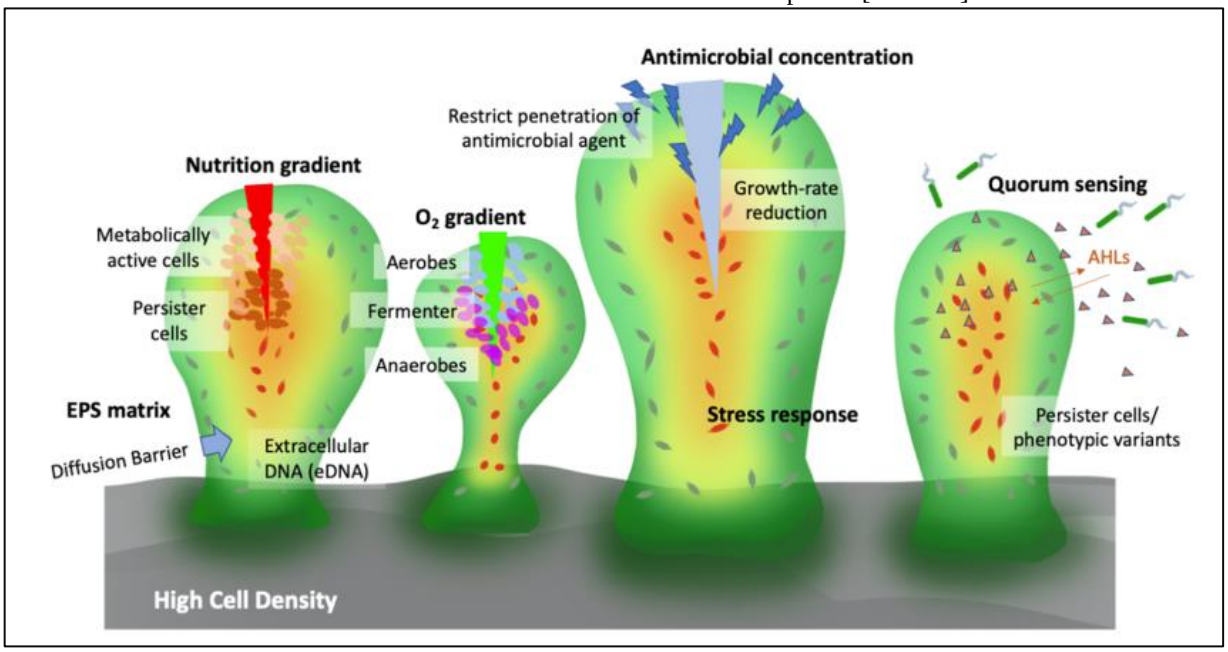

Figure 3: Biofilm Resistance Mechanisms.

Clarifying Definitions in Biofilm Resistance

To better understand biofilm resistance, it is essential to clarify certain definitions. "Antibiotic resistance" refers to the inherited ability of bacterial cells, through genetic mutations, to survive and multiply despite exposure to antibiotics. This resistance, resulting from permanent genetic modifications, is well-documented in planktonic cells and includes mechanisms such as alterations in antibiotic targets, enzymatic inactivation of antibiotics, and increased efflux pump activity. The term "adaptive resistance," as described by de la Fuente-Núñez et al. , refers to temporary genetic alterations that lead to resistance in biofilm bacteria, which disappears once the bacteria revert to a planktonic state. In discussing biofilm resistance, the term generally encompasses bacterial resistance, regardless of its permanence [100-110].

Biofilm Heterogeneity: Concentration Gradients

Biofilms exhibit clear stratification in bacterial metabolic activity due to varying concentrations of nutrients and oxygen available to surface cells and those deeper within the biofilm. Research by Sternberg et al. utilized fluorescent tags to monitor specific metabolites, demonstrating that cells at the center of biofilms exhibit reduced growth activity compared to those at the bulk liquid interface. This growth activity can be restored by providing appropriate nutrients, indicating the critical role of nutrient availability in biofilm

metabolic activity. Further research by de Beer and colleagues constructed oxygen concentration profiles across biofilms, revealing that oxygen levels decreased by as much as 30-fold in the center of larger microcolonies . These findings suggest that nutrient and oxygen depletion towards the biofilm's center leads to stratified metabolic activity, growth rates, and gene expression. As antibiotics like β-lactams target dividing bacterial cells, they are less effective against the more dormant cells within the biofilm [100-110].

Stress Responses Triggered by Nutrient Limitation

Nutrient limitation not only alters bacterial growth activity but also triggers stress responses that enhance antibiotic tolerance or resistance. Recent studies suggest that nutrient limitation-induced antibiotic tolerance is not merely a consequence of reduced metabolic activity but is instead governed by complex regulatory pathways. The stringent response has been implicated in increased antibiotic tolerance in nutrient-starved *Pseudomonas aeruginosa* and fluoroquinolone tolerance in *Escherichia coli* biofilms . Additionally, survival and heat shock responses have been linked to fluoroquinolone and aminoglycoside resistance in planktonic *Pseudomonas* , though further investigation is needed to understand their role in biofilm-associated resistance [100-113].

Persister Cells and Antibiotic Treatment Challenges

A subpopulation of bacterial cells known as "persister cells," which are genetically identical to active cells but exhibit a more dormant and antibiotic-tolerant physiological state, pose significant challenges in biofilm-associated infections. Persister cells, often present in exponentially growing bacterial populations before antibiotic treatment, are considered an adaptive strategy for coping with environmental changes, allowing them to resume growth once stress is alleviated. These cells have been identified in several bacterial species, including *Mycobacteria* and *Borrelia*, and are recognized as a major resistance mechanism in *Staphylococcus epidermidis* biofilms . Their presence complicates antibiotic treatment, as different phenotypes are proven to exist. State-of-the-art strategies to combat persister cells involve sensitizing them by introducing specific carbon sources and terminal electron acceptors [100-113].

Roles of the EPS Matrix: Diffusion Barrier

The EPS matrix of biofilms was initially believed to confer resistance by reducing antibiotic penetration. Suci and colleagues investigated the impact of the EPS matrix on antibiotic penetration using a germanium crystal substratum in an infrared (IR) field, demonstrating that while the biofilm significantly reduced antibiotic penetration, it did not entirely block it . The penetration rate of antibiotics through biofilms depends on their chemical nature and does not directly account for biofilm recalcitrance. However, the diffusion barrier plays a crucial role in accumulating and retaining enzymes that degrade antibiotics within the extracellular matrix. For example, β-lactamase, overproduced by *P. aeruginosa* biofilms, may reduce the functionality of β-lactams by degrading them before they reach bacterial cells [100-113].

Additional Resistance Mechanisms Induced by Extracellular DNA

Extracellular DNA (eDNA) in the EPS matrix serves as structural support for biofilms and has been implicated in enhancing resistance by inducing additional resistance mechanisms. Mulcahy et al. demonstrated that eDNA-induced antibiotic resistance involves cation gradients and the release of genomic DNA. eDNA can chelate cations that stabilize lipopolysaccharide and the outer membrane, leading to cell lysis and increased DNA concentration in the biofilm matrix. This cation limitation induces the PhoPQ- and PmrAB-regulated cationic antimicrobial peptide resistance operon PA3552−PA3559 in *P. aeruginosa*, significantly increasing resistance to cationic antimicrobial peptides and aminoglycosides without affecting β-lactam and fluoroquinolone resistance [100-113].

High Cell Density, Quorum Sensing, and Mutation

Bacteria in biofilms live in high-density and close-proximity environments, which have been suggested to contribute to their enhanced resistance to antibiotics. Larsen tested planktonic *Porphyromonas gingivalis* susceptibility to amoxicillin, doxycycline, and metronidazole at cell densities comparable to those in biofilm populations (107 to 108 cells/mL). The results showed increased minimum inhibitory concentrations (MICs) for planktonic cultures at these densities, suggesting an inoculum effect on biofilm resistance. The molecular mechanism behind this inoculum effect is speculated to be quorum sensing (QS). QS enables bacteria to sense and respond to cell density changes through various regulations, influencing biofilm development and regulating the production of virulence factors such as extracellular enzymes and cellular lysins, which are critical for phagocytosis resistance in *P. aeruginosa* biofilms. QS inhibitors have been proposed as a strategy to overcome biofilm resistance [100-113].

Increased Mutability and Horizontal Gene Transfer

Biofilms exhibit increased mutability and horizontal gene transfer compared to planktonic states . This increased mutability is associated with heightened oxidative stress within biofilms. The production of endogenous reactive oxygen species, combined with oxidative bursts from the immune system and insufficient antioxidant defenses, leads to

increased oxidative stress. This stress is linked to hypermutable *P. aeruginosa* strains observed in cystic fibrosis patients. Boles and Singh found that endogenous oxidative stress causes double-stranded DNA breaks, which are repaired via mutagenic mechanisms involving recombinatorial DNA repair genes, generating genetic variants. The addition of antioxidants has been shown to reduce the occurrence of genetic variants in biofilms [100-113].

New Strategies to Combat Biofilms:

The inefficacy of traditional therapeutic methods underscores the need for enhanced approaches in biofilm treatment [128]. Novel strategies are essential to address challenges associated with biofilm formation, such as antibiotic resistance and high pathogenicity. Implants and other foreign bodies play a critical role in the development of biofilm-related infections [129]. Effective treatment of these infections often necessitates the removal or replacement of contaminated medical devices, coupled with the administration of potent antibiotics. In cases where device removal is unfeasible, prolonged antibiotic therapy is advised to inhibit biofilm growth [130]. Research suggests that mature biofilms are more difficult to treat compared to premature ones, largely due to inadequate early diagnosis, which allows biofilms to mature within the body and cause clinical complications [131]. Selecting antibiotics for biofilm treatment requires consideration of their ability to penetrate the biofilm matrix and their sensitivity to the biofilm bacteria [4]. Studies reveal that biofilm-associated bacteria exhibit greater antibiotic resistance than planktonic cells [132]. Hence, combinatorial therapy, which utilizes multiple agents with different mechanisms of action, proves more effective than monotherapy. For instance, one agent may target actively growing cells while another targets dormant cells [133]. Proper dosing and timing are critical for the success of such therapies.

Recent developments have focused on preventing biofilm formation, with antimicrobial or antifouling surfaces emerging as a promising area of research [134,135]. Polyethylene glycol (PEG) coatings, for example, are designed to reduce microbial adhesion [134,136]. Additionally, the development of polyurethane polymers impregnated with antibiotics or disinfectants has been explored to create antimicrobial surfaces [136,137]. Nanoparticle coatings, such as those containing silver, offer antioxidant and antibacterial properties that can inhibit biofilm formation [138,139]. However, these surface coatings face challenges like erosion and leaching, which may still permit biofilm development. Emerging strategies also include the creation of anti-biofilm compounds or biofilm dispersal methods [140]. A variety of molecules, including peptides, enzymes, polyphenols, and specific antibiotics, have shown potential as anti-biofilm agents [141]. Some of these agents disrupt bacterial signaling pathways, particularly in both Gram-positive and Gram-negative bacteria, thereby hindering biofilm formation.

Since biofilm formation contributes significantly to bacterial pathogenicity and antibiotic resistance, targeted strategies are crucial for managing this issue. Removal and replacement of infected implants, combined with aggressive antibiotic therapy, are often necessary [6]. When removal is not an option, long-term antibiotic administration is essential to prevent biofilm proliferation. The efficacy of premature biofilm treatment highlights the importance of early detection, as delayed diagnosis can lead to the maturation of biofilms and subsequent clinical issues [127]. Antibiotic selection should prioritize both sensitivity and penetration capabilities [6], as biofilm bacteria are more resistant than planktonic cells, making combinatorial therapy preferable [128]. This approach involves using multiple agents that target different aspects of bacterial life, such as dormant versus actively growing cells. Proper antibiotic dispensation in terms of dosage and timing is also vital. Antifouling or antimicrobial surfaces represent another preventive strategy against biofilm formation [129]. PEG-based hydrophilic coatings, for example, inhibit microbial adhesion, while polyurethane polymers loaded with antibiotics create antimicrobial surfaces [130, 131]. Nanoparticle coatings, including those with silver or antioxidants, further prevent biofilm formation [69, 132]. However, erosion and leaching remain significant challenges for these coating strategies.

Photodynamic therapy (PDT) has also been explored for preventing wound biofilm infections by using photoactive dyes and irradiation to kill bacteria, though care must be taken to protect surrounding tissues and avoid laser exposure to patients' eyes [133]. Anti-biofilm molecules, such as peptides, enzymes, and polyphenols, can disrupt bacterial signaling pathways, effectively inhibiting biofilm formation and offering promising therapeutic options [134]. In the realm of biofilm inhibition, various strategies have been proposed, including the disruption of AHL-mediated quorum sensing, the inhibition of bacterial stringent responses, and the enzymatic breakdown of extracellular polysaccharides. For instance, certain synthetic halogenated furanones have been shown to interfere with bacterial signaling and biofilm formation by competing with AHL molecules [135, 139]. Additionally, peptides like 1018 inhibit biofilm formation by disrupting alarmone accumulation during bacterial stress responses, a critical mechanism for biofilm maintenance [134, 135].

Enzymatic approaches, such as the use of DNase I and Dispersin B, target the extracellular matrix of biofilms, effectively exposing bacteria to antimicrobial agents [140-152]. Tannic acid and other polyphenolic compounds inhibit biofilm

formation by cleaving peptidoglycan, a key component of bacterial cell walls [152-160]. Furthermore, bacteriophage-derived endolysins offer a species-specific approach to cleaving peptidoglycan and disrupting biofilms, even in antibiotic-resistant strains [161-166]. Biofilm disassembly, a process involving the degradation of the extracellular matrix and changes in cellular physiology, is also a promising area of research. The accessory gene regulatory (agr) system, found in various bacteria, plays a role in producing matrix-degrading enzymes and preventing biofilm maturation [167-178]. Understanding and manipulating these processes offer new avenues for combating biofilm-related infections.

The alteration of membrane potential or permeabilization is another key mechanism by which antimicrobial peptides exert their effects. This process leads to the disruption of the cytoplasmic membrane through pore formation via various mechanisms, including the barrel-stave model , toroidal pore formation , or a non-pore carpet-like mechanism , ultimately resulting in the efflux of intracellular contents. Lantibiotics, a class of peptide antibiotics characterized by their ring structure linked through thioester bonds involving lanthionine and methylanthionine, or unsaturated amino acids such as dehydroalanine or 2-amino isobutyric acid, play a significant role in this process. These peptides, synthesized by ribosomes and post-translationally modified in Gram-negative bacteria, serve as anti-biofilm agents. Their intramolecular ring structure allows them to inhibit a broad spectrum of bacteria . Lantibiotics exert their antibacterial effects by compromising the bacterial membrane, thereby inhibiting enzyme production. The most renowned lantibiotic, nisin, forms a complex with lipid I and II, inhibiting cell wall biosynthesis . Nisin also increases membrane permeability by forming short-lived pores . Another pore-forming lantibiotic, subtilin, similar in structure to nisin, dissipates the transmembrane proton motive force, causing the release of cytoplasmic solutes from Staphylococcus simulans, B. subtilis, and membrane vesicles. Subtilin interacts with bactoprenyl pyrophosphate, causing membrane permeabilization in a lipid II-dependent manner. In vitro modifications have successfully introduced thioester rings into various biologically active peptides, suggesting that clinically modified lantibiotics could be used after thorough in vivo testing . Epidermin and gallidermin, which share a lipid II binding motif with nisin but differ in size (22 amino acids compared to nisin's 34), also disrupt lipid II biosynthesis and interact with lipid-I, lipid-II, and their intermediates, leading to bacterial death. Studies indicate that gallidermin efficiently inhibits biofilm formation by Staphylococci, likely by repressing genes involved in biofilm formation, such as atl (major autolysin) and ica (intercellular adhesin). However, its effect on mature biofilms (24-hour and 5-day-old) is significantly diminished [179-190].

Biosurfactants, amphipathic molecules with antibacterial properties, inhibit bacterial cell-surface adhesion and biofilm formation. Sophorolipids, a class of biosurfactants, disrupt bacterial membranes by increasing permeability. In B. subtilis, sophorolipids disrupt bacterial cells and release the intracellular enzyme malate dehydrogenase, leading to cytoplasmic content efflux. They also inhibit biofilm formation by single or mixed cultures of B. subtilis and S. aureus at very low concentrations. This suggests that sophorolipids could be used as adjuvants with other antibacterial agents to inhibit bacterial growth or disassemble biofilms . Biofilms can also be eradicated using polyhexamethylene biguanide, a cationic antimicrobial agent that disrupts membrane permeability without lysing the cell wall. Chlorhexidine alters cell osmolarity by binding to negatively charged components. Compared to these agents, penta-silver hexaoxoiodate (Ag(5)IO(6)) is more effective in killing a broad spectrum of planktonic organisms, inhibiting microbial adhesion for extended periods, and dismantling mature biofilms of C. albicans, P. aeruginosa, and S. aureus. The high efficacy of this nanomaterial may be due to its structure, which contains both cationic and anionic silver, with iodate-protected anions. This compound is a potential antimicrobial agent for disinfecting medical devices such as catheters, implants, ventilators, and wound dressings [191-204].

The process of cell division is critical for the survival of bacteria within biofilms and their subsequent spread to new areas. Silver accumulates within intracellular vacuoles, damaging the plasma membrane and altering the electric potential, thereby preventing cell division . Some antimicrobial peptides function by inhibiting cytoplasmic proteins essential for cell division and survival. These peptides penetrate the bacterial cytosol through either the flip-flop method or channel formation in the outer membrane protein. Notably, certain antibacterial peptides are rich in proline, such as pyrrhocoricin, apidaecin , and drosocin . These peptides bind to the multi-helical lid region of DnaK, a bacterial heat shock protein, interfering with the initiation of chromosomal DNA replication. They also disrupt the interaction between DnaK and DnaJ, leading to bacterial death. Pyrrhocoricin enters the bacterial cytosol via its C-terminus, while its N-terminus inhibits the ATPase activity of DnaK . Additionally, proline-rich AMPs actively enter bacterial cells and interfere with translation initiation by binding to the ribosome tunnel . Microcin B17, a ribosomally synthesized antimicrobial peptide from Enterobacteriaceae, inhibits DNA gyrase, thereby hindering DNA replication. It is also the first peptide

capable of inhibiting a type II DNA topoisomerase . Moreover, chelating agents like EDTA can destabilize biofilms by sequestering essential ions such as iron, zinc, magnesium, and calcium, making them suitable for biofilm management . Chitosan, a natural polymer with cationic properties, can disrupt negatively charged cell membranes as soon as microbes settle on the surface [105-116].

Certain classes of antimicrobial peptides (AMPs) kill bacteria through direct interactions with nucleic acids without causing membrane permeabilization, such as Buforin II . The antimicrobial peptide PR-39, isolated from pig intestine, penetrates the outer membrane and halts the synthesis of DNA and proteins, the fundamental components of biofilms . Another peptide, indolicidin, permeabilizes the membrane without lysing bacterial cells. It also inhibits DNA synthesis and exhibits specific binding to DNA rather than RNA . Studies have reported that LL-37, a human host defense peptide, reduces bacterial adhesion and promotes type IV pili-mediated twitching motility. LL-37 also down-regulates quorum-sensing-related genes . It has been found effective against S. epidermidis by inhibiting bacterial attachment and subsequent biofilm formation . Citropin (from the green tree frog *Litoria citropa*) and melimine (a non-hemolytic hybrid peptide) have potent activity against *P. aeruginosa* and *S. aureus* without toxic effects in animal models, suggesting their use in preventing bacterial adhesion on medical devices like catheters and contact lenses. Cadexomer iodine, another modified peptide, binds with cytoplasmic membrane proteins and penetrates bacterial cells, inhibiting protein synthesis, disrupting lipid membranes, and interfering with nucleic acid function . Recent studies have shown that AMPs can coat bacteria or biomaterial surfaces, reducing bacterial adhesion and biofilm formation . Bacteriocins such as bovicin HC5 (produced by *Streptococcus bovis* HC5) and nisin alter the hydrophobicity of surfaces, minimizing bacterial adhesion to food items, which may be more effective than eradicating established biofilms. This property is beneficial for the long-term storage and preservation of packaged foods . Pili or fimbriae, long filamentous surface structures that facilitate bacterial adherence to host tissues, are also involved in biofilm formation. Components like PilB and PilA are critical for biofilm formation, though not PilC . Pili are classified into two groups: Type I pili, composed mainly of FimA and FimH, the latter being a mannose-binding adhesion component that facilitates bacterial invasion . Most uropathogenic *Escherichia coli* (UPEC) possess type I pili with FimH adhesin, enabling colonization on silicone implants and urinary bladder surfaces, leading to catheter-associated urinary tract infections (CAUTI) . After entering host cells, this pathogen evades the immune system and forms large intracellular bacterial communities (IBC), similar to biofilms . Lactoferrin, a peptide found in gingival crevicular fluids and saliva, inhibits the attachment of *S. mutans* and *Streptococcus gordonii*, preventing biofilm formation in the oral cavity . Studies also suggest that lactoferrin prevents biofilm formation by *Porphyromonas gingivalis* and *Prevotella intermedia* in subgingival plaque at concentrations as low as ≥8 μg/ml [216-258].

Conclusion:

Biofilms represent a significant challenge in medical settings due to their inherent resistance to antibiotics and their role in persistent infections. The complex structure of biofilms, characterized by heterogeneity in metabolic activity, nutrient limitation, and stress responses, significantly contributes to their resilience. Additionally, the presence of persister cells, the diffusion barrier posed by the extracellular polymeric substance (EPS) matrix, and the involvement of extracellular DNA further complicate treatment efforts. Emerging strategies to combat biofilms include disrupting quorum sensing, enhancing antibiotic penetration, and employing novel antimicrobial agents like nanoparticles and biosurfactants. The development of anti-biofilm surfaces and coatings, along with the use of antimicrobial peptides and bacteriophages, also offers promising avenues for preventing and treating biofilm-associated infections. These strategies are essential in overcoming the limitations of traditional antibiotics and ensuring the effective management of biofilm-related infections. However, the complexity of biofilms necessitates a multifaceted approach that combines early detection, targeted therapy, and the prevention of biofilm formation to mitigate the impact of these resilient bacterial communities in clinical settings.

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