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From Surface to Survival – Microbial Biofilms: A Hidden Threat in Therapeutics

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Abstract: Biofilms represent a highly organized and adaptive mode of microbial existence in which microorganisms form surface-associated communities encased within a self-produced extracellular polymeric substance (EPS) matrix. This structural organization promotes physiological heterogeneity, metabolic cooperation, and coordinated gene regulation, enhancing resilience against environmental stressors, antimicrobial agents, and host immune defenses. In clinical, veterinary, food-processing, and environmental contexts, biofilms play a central role in chronic infections, device-associated infections, biofouling, and contamination of production systems. Biofilm recalcitrance is further strengthened by persister cells, altered growth kinetics, and diffusion barriers created by the EPS matrix. This review synthesizes current knowledge on biofilm composition, architecture, regulatory mechanisms, antimicrobial tolerance, and detection methodologies. Understanding biofilm biology is essential for developing innovative strategies to control biofilm-associated challenges across medical, veterinary, and industrial domains.

Keywords: Biofilm architecture, Quorum sensing regulation, Antimicrobial resistance and tolerance, Persister cells, Biofilm detection methodologies.

Introduction

According to comprehensive reviews and National health data, up to 65% of nosocomial infections and approximately 80% of chronic infections in humans are biofilm-related, indicating that most difficult to treat infections involve biofilm communities rather than planktonic cells. This high prevalence underlies the importance of understanding biofilms for effective therapy. The National Institutes of Health (NIH) reported that biofilms are responsible for an estimated 80% of all microbial infections in humans, underscoring their ubiquitous role in chronic disease processes. The consequences of biofilm-mediated infections extend beyond clinical failure to significant economic burden. Public health relevance is also underscored by the fact that many biofilm-associated infections in animals are zoonotic and linked with environmental contamination that have impact on One Health.

Biofilms are structured community of microorganisms that are irreversibly attached to biotic or abiotic surfaces and embedded within a self-produced extracellular polymeric substance (EPS) matrix. Unlike planktonic cells, microorganisms within biofilms exhibit altered phenotypic traits, particularly in growth rate, gene expression, and metabolic activity, which confer enhanced survival advantages in diverse environments [9]. Biofilm formation is now recognized as a predominant mode of microbial life, occurring in natural, industrial, food, and clinical settings, including veterinary and human health sectors [20].

The EPS matrix is a defining feature of biofilms and is primarily composed of polysaccharides, proteins, lipids, and extracellular DNA and their percentage of composition. This matrix provides structural stability, mediates adhesion to surfaces, and facilitates intercellular communication among microbial cells [21].

Biofilm development is a dynamic and multistep process involving initial reversible attachment, irreversible adhesion, microcolony formation, maturation, and eventual dispersion. These stages are tightly regulated by environmental cues and complex regulatory networks, including quorum sensing systems that enable microbial populations to coordinate gene expression in a cell density–dependent manner. Such coordinated behaviour allows biofilm communities to adapt efficiently to nutrient availability, shear forces, and host-associated stresses [31].

From clinical perspective, biofilms are of particular concern due to their strong association with chronic and recurrent infections. Biofilm-forming bacteria and fungi exhibit markedly increased resistance to antimicrobial agents compared to their planktonic counterparts, often requiring concentrations many times higher than the minimum inhibitory concentration for eradication. This resistance is multifactorial, involving limited drug diffusion, altered metabolic states, and the presence of persister cells within the biofilm population [32]. In food safety and veterinary public health, biofilms formed on processing equipment, animal housing surfaces, and water systems serve as persistent reservoirs of pathogenic microorganisms. These biofilms contribute to contamination of food of animal origin and pose significant challenges to hygiene and sanitation practices. Understanding the molecular basis and ecological significance of biofilm formation is therefore essential for developing effective control strategies in both clinical and agricultural contexts [9].

Beyond surface attachment, biofilm formation represents a survival strategy that enables microorganisms to withstand fluctuating environmental and host-associated stresses. Within a biofilm, cells exhibit cooperative behaviour, sharing nutrients and genetic material through horizontal gene transfer, which enhances adaptability and long-term persistence. This communal lifestyle provides a selective advantage over planktonic growth, particularly in hostile environments such as nutrient limitation, desiccation, oxidative stress, and antimicrobial exposure [30].

The transition from planktonic to biofilm mode of growth is accompanied by extensive changes in gene expression profiles. Genes involved in motility are often downregulated, while those responsible for adhesion, EPS synthesis, stress tolerance, and virulence are upregulated. These transcriptional shifts are regulated by global regulatory systems and secondary messengers such as cyclic di-GMP, which plays a central role in promoting biofilm maturation and inhibiting dispersal. Such regulatory plasticity underscores the complexity of biofilm biology and its relevance in microbial pathogenesis [19].

Another important characteristic of biofilms is their ability to evade host immune defenses. Phagocytosis and antibody-mediated clearance are significantly impaired due to the protective EPS matrix and the altered antigenic expression of biofilm-associated cells. Inflammatory responses triggered by biofilms often fail to eliminate the infection and instead contribute to tissue damage and chronicity. This immune evasion explains the persistent nature of many biofilm-associated infections despite an apparently competent host immune system [21].

Biofilms are rarely composed of a single microbial species; instead, they often exist as multispecies communities where interspecies interactions influence structure, metabolism, and pathogenicity. Synergistic relationships within mixed biofilms can enhance overall resistance to antimicrobials and environmental stressors, while antagonistic interactions may shape microbial dominance. Such polymicrobial biofilms are particularly relevant in veterinary infections, food-processing environments, and natural ecosystems, where diverse microbial populations coexist on shared surfaces [36]. Understanding this baseline is essential because it reframes therapy goals from simply "kill the organism" to "disrupt a structured, heterogeneous community and its protective matrix."

Composition of biofilms

Biofilms are highly organized microbial assemblages in which cells are embedded within a self-generated extracellular polymeric substance (EPS) matrix that anchors them firmly to living or non-living surfaces. This EPS matrix is the dominant structural component of biofilms and governs their physical stability, spatial arrangement, and resistance to environmental challenges, clearly differentiating biofilm-associated microorganisms from planktonic cells . Additionally, the EPS acts as a physical and chemical barrier that restricts the penetration of antimicrobial agents and host immune factors, thereby contributing significantly to the persistence of biofilm-associated infections (Fig. 1).

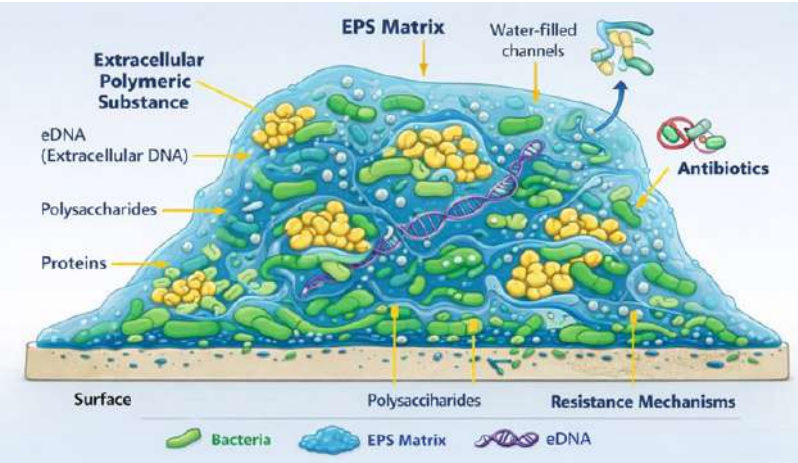

Figure 1: Structural organization of a biofilm showing bacterial cells embedded within the extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, extracellular DNA, and water channels.

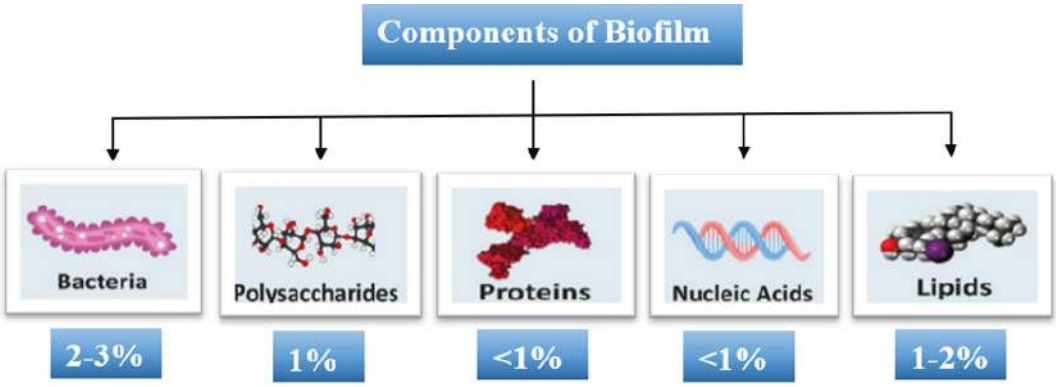

Figure 2: Major components of the biofilm matrix and their approximate proportional contributions to structural stability and function.

The microbial population within biofilms is composed of bacterial, fungal, or mixed-species cells arranged in discrete clusters. These cells exist in varied physiological states, including active, slow-growing, and dormant forms, allowing metabolic cooperation and survival under nutrient limitation and antimicrobial pressure. Such heterogeneity enhances adaptability and persistence in diverse ecological niches [43]. Polysaccharides constitute the primary macromolecular framework of the EPS matrix and are essential for surface attachment, intercellular cohesion, and three-dimensional biofilm development. The chemical nature of these polymers differs among microorganisms, influencing biofilm elasticity, permeability, and tolerance to physical and chemical stresses [42]. Proteins are integral components of the biofilm matrix, fulfilling both structural and functional roles. These include adhesive proteins that promote stable surface binding, as well as extracellular enzymes that participate in nutrient acquisition and matrix modification. The dynamic turnover of matrix proteins plays a key role in biofilm maturation and dispersal processes [24].

Extracellular DNA (eDNA) serves as a critical scaffold within biofilms, contributing to early attachment and overall matrix stability through electrostatic interactions. In addition to its structural function, eDNA facilitates genetic exchange among biofilm-associated cells, thereby enhancing adaptability and the spread of antimicrobial resistance traits [5].

Lipids and amphipathic molecules, though less abundant, influence the hydrophobic properties of the biofilm matrix and affect interactions with antimicrobial agents and disinfectants. These molecules are also involved in the formation of extracellular membrane vesicles, which function in intra-biofilm transport of enzymes, toxins, and signaling compounds [43]. Divalent and trivalent ions such as calcium, magnesium, and iron contribute significantly to biofilm integrity by cross-linking matrix polymers and strengthening EPS cohesion. These ions enhance mechanical stability and play an important role in biofilm persistence under shear stress and fluctuating environmental conditions [22].

Water represents the most abundant component of the biofilm matrix and is organized into interconnected channels that allow efficient transport of nutrients, oxygen, and metabolic waste. This channel network supports microbial viability in mature biofilms by functioning as an internal distribution system within the matrix [17].

Microorganisms Capable of Causing Biofilm

Several Gram-positive bacteria are well-known biofilm formers, particularly in clinical and hospital environments. Staphylococcus aureus and Staphylococcus epidermidis are among the most studied biofilm-forming organisms due to their role in device-associated infections. Other Gram-positive bacteria such as Enterococcus faecalis, Streptococcus mutans, Listeria monocytogenes, and Bacillus spp. also demonstrate strong biofilm-forming ability on biotic and abiotic surfaces [20]. Gram-negative bacteria are prominent biofilm formers in clinical, environmental, and industrial settings. Pseudomonas aeruginosa is a model organism for biofilm research and is strongly associated with chronic infections. Other significant biofilm-forming Gram-negative bacteria include *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Salmonella enterica, Vibrio cholerae, Campylobacter jejuni, and Helicobacter pylori* [23].

Opportunistic pathogens capable of surviving in hospital environments possess strong biofilm-forming properties. These include *Burkholderia cepacia complex, Serratia marcescens, Proteus mirabilis, Citrobacter spp., and Providencia spp.* Biofilm formation in these organisms contributes to persistence on medical devices and resistance to disinfectants and antimicrobials [31]. Fungi, particularly yeasts, are increasingly recognized as important biofilm formers. *Candida albicans* is the most extensively studied fungal biofilm former, followed by non-albicans species such as *Candida tropicalis, Candida glabrata, Candida parapsilosis, and Candida krusei*. Other fungi such as *Aspergillus fumigatus, Cryptococcus neoformans,* and *Trichosporon spp*. are also capable of forming biofilms, especially in immunocompromised hosts [35].

Candida biofilms readily develop on intravascular catheters, prosthetic devices, and mucosal surfaces, exhibiting high resistance to azoles and other antifungal agents. These biofilms are strongly associated with candidemia and invasive candidiasis, especially in critically ill and immunosuppressed patients, resulting in high morbidity and mortality rates [4].

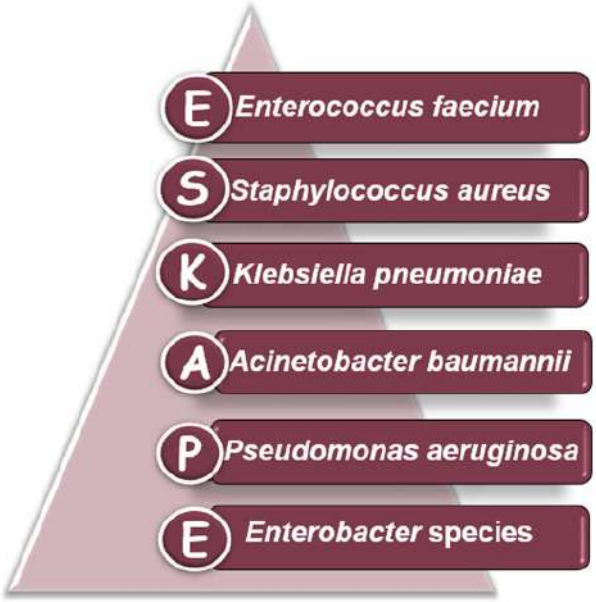

Figure 3: ESKAPE pathogens demonstrating biofilm-associated virulence and antimicrobial resistance mechanisms.

Non-tuberculous Mycobacteria such as Mycobacterium avium, Mycobacterium abscessus, and Mycobacterium fortuitum are capable of forming robust biofilms in water systems and medical devices. Biofilm formation contributes to their persistence in hospital water supplies and resistance to disinfectants, posing a risk for nosocomial infection [21]. Anaerobic bacteria involved in oral, periodontal, and gastrointestinal infections frequently form biofilms. Species such as Porphyromonas gingivalis, Fusobacterium nucleatum, Bacteroides fragilis, and Clostridium spp. are key contributors to polymicrobial biofilms, particularly in chronic and inflammatory conditions [15].

In food and environmental microbiology, they pose serious contamination risks. Common biofilm formers include Listeria monocytogenes, Salmonella spp., Escherichia coli O157:H7, Staphylococcus aureus, Vibrio spp., and Pseudomonas spp. These organisms readily colonize food-processing surfaces and resist sanitation procedures [10]. Many biofilms are polymicrobial in nature, involving complex interactions between bacteria–bacteria, bacteria–fungi, or fungi–fungi. Such mixed-species biofilms exhibit enhanced structural stability, metabolic cooperation, and increased tolerance to antimicrobials compared to single-species biofilms. Polymicrobial biofilms are commonly observed in chronic wounds, dental plaque, and indwelling medical devices [37].

Biofilm Formation

Biofilm formation is a regulated, sequential process through which microorganisms transition from a free-living planktonic state to a surface-associated, multicellular community.

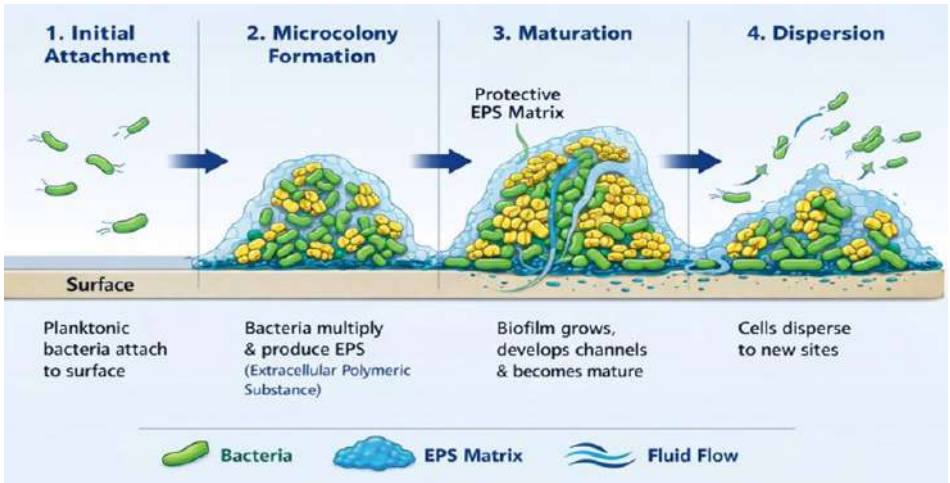

Figure 4: Sequential stages of biofilm development: initial attachment, irreversible adhesion, microcolony formation, maturation, and dispersion.

Biofilm development is a dynamic and regulated process consisting of:

1. Initial reversible attachment,
2. Irreversible adhesion
3. Microcolony formation
4. M aturation
5. Dispersion

This process is driven by environmental cues such as nutrient availability, surface properties, hydrodynamic conditions, and host-associated factors. The ability to form biofilms allows microorganisms to colonize diverse niches and enhances long-term survival under adverse conditions [31].

1. Initial Attachment

1.1. Surface Conditioning

The first step in biofilm formation involves surface conditioning, where organic and inorganic molecules from the surrounding environment rapidly adsorb onto biotic or abiotic surfaces. These conditioning films modify surface characteristics such as charge, hydrophobicity, and roughness, thereby influencing subsequent microbial attachment. In biological systems, host-derived proteins and polysaccharides play a critical role in determining microbial adhesion efficiency [8].

1.2. Reversible and irreversible attachment

Following surface conditioning, planktonic microorganisms approach the surface through Brownian motion, sedimentation, or active motility. Initial attachment at this stage is weak and reversible, mediated by non-specific physicochemical forces such as van der Waals interactions, electrostatic forces, and hydrophobic interactions. At this point, microbial cells can still detach easily in response to shear forces or unfavorable conditions [14].

Irreversible attachment marks a critical transition in biofilm development and is characterized by the establishment of strong cell–surface interactions. Microorganisms express specific adhesins, fimbriae, pili, and surface-associated proteins that anchor cells firmly to the substrate. Concurrently, the synthesis of extracellular polymeric substances begins, reinforcing attachment and committing cells to a sessile lifestyle [30].

2. Microcolony Formation

Once irreversibly attached, microbial cells undergo localized proliferation and recruit neighbouring cells to form microcolonies. These early biofilm structures represent organized clusters rather than random cell aggregates. During this stage, cell–cell adhesion increases and the production of EPS intensifies, providing structural cohesion and protection against environmental stresses [34]. This is solely regulated by a bacterial communication mechanism called as quorum sensing.

3. Quorum Sensing: Molecular mechanisms across Bacterial group

Quorum sensing (QS) is a population density dependent regulatory system that enables bacteria to coordinate collective behaviours through the production, release, accumulation, and detection of small signalling molecules known as autoinducers. QS regulates a wide spectrum of physiological functions, including biofilm formation, virulence expression, motility, sporulation, and stress adaptation. Although the underlying principle of QS is conserved, the molecular architecture, signal chemistry, and regulatory circuits vary significantly among bacterial taxa [30].

AHL-Based Systems (LuxI/LuxR Paradigm)

In Gram-negative bacteria, QS is predominantly mediated by *N*-acyl homoserine lactones (AHLs). The canonical QS system comprises a signal synthase (LuxI-type protein) and a cognate cytoplasmic receptor (LuxR-type transcriptional regulator). LuxI catalyzes the synthesis of AHLs using S-adenosylmethionine and acyl-acyl carrier protein as substrates. As bacterial population density increases, AHLs accumulate in the extracellular milieu and diffuse back into cells, where they bind LuxR. The AHL–LuxR complex undergoes conformational stabilization, enabling it to bind promoter regions of QS-regulated genes and activate or repress transcription [21]. This system was first characterized in *Vibrio fischeri*, where QS regulates bioluminescence. However, variations of the LuxI/LuxR circuitry are widely distributed among Gram-negative pathogens and environmental bacteria [47].

Hierarchical QS Networks

Pseudomonas aeruginosa exhibits one of the most complex QS architectures, consisting of multiple interconnected systems Las, Rhl, and Pqs. The Las system (LasI/LasR) occupies the top of the hierarchy and regulates the Rhl system (RhlI/RhlR), while the Pqs system utilizes alkyl quinolone signals such as PQS (2-heptyl-3-hydroxy-4-quinolone). These systems collectively regulate elastase production, rhamnolipid synthesis, pyocyanin expression, and biofilm maturation. Crosstalk among QS circuits ensures temporal regulation of virulence and metabolic functions [45]. Disruption of QS in *P. aeruginosa* significantly attenuates pathogenicity without directly affecting growth, highlighting QS as a promising anti-virulence target [32].

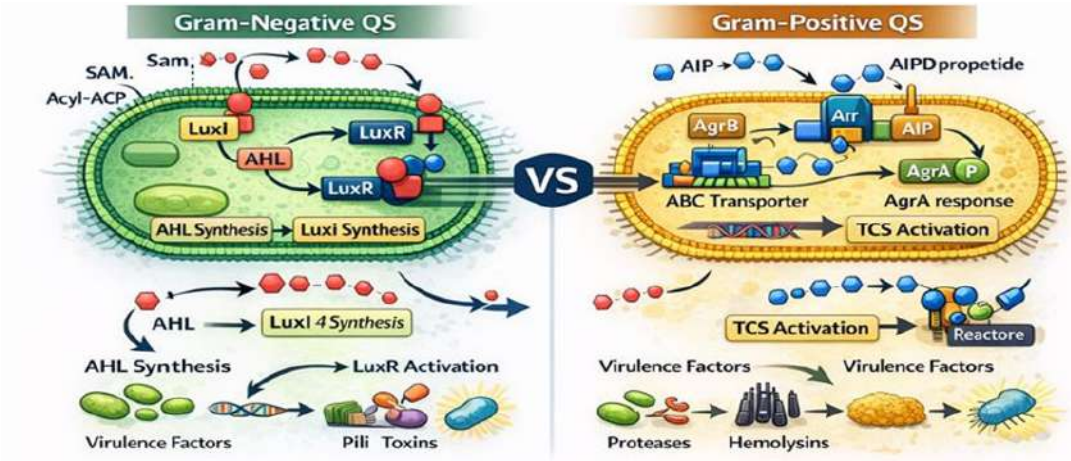

Figure 5: Quorum sensing pathways in bacteria: (A) AHL-mediated signaling in Gram-negative bacteria; (B) peptide-mediated signaling via two-component systems in Gram-positive bacteria.

Autoinducing Peptides and Two-Component Systems

Gram-positive bacteria primarily employ ribosomally synthesized autoinducing peptides (AIPs) as QS signals. These peptides are processed and exported via ATP-binding cassette transporters. Upon reaching a threshold concentration, AIPs are detected by membrane-bound histidine kinase receptors, triggering phosphorylation cascades through two-component regulatory systems. The phosphorylated response regulator subsequently modulates transcription of QS-controlled genes [29].

Agr QS System

The accessory gene regulator (agr) system in Staphylococcus aureus is a well-characterized peptide-based QS network. The agr locus encodes AgrD (AIP precursor), AgrB (processing/export protein), AgrC (sensor kinase), and AgrA (response regulator). Accumulated AIP binds AgrC, leading to AgrA phosphorylation, which activates transcription from the P2 and P3 promoters. This results in upregulation of RNAIII, a regulatory RNA molecule that controls the expression of numerous virulence factors, including hemolysins and proteases, while repressing surface adhesins. The agr system thus orchestrates a switch from colonization to invasive behavior.

In *Bacillus subtilis*, QS involves multiple peptide pheromones (ComX, CSF, Phr peptides) that regulate competence development, sporulation, and biofilm formation. The ComX pheromone is detected by the ComP/ComA two-component system, leading to activation of genes involved in DNA uptake and community behaviour. This QS network integrates environmental signals with population density cues, enabling adaptive decision-making at the community level [19].

Autoinducer-2 (AI-2): Interspecies Quorum Sensing

AI-2 is a furanosyl borate diester derived from the LuxS enzyme-mediated activated methyl cycle. Unlike AHLs and AIPs, AI-2 is produced by both Gram-negative and Gram-positive bacteria, making it a universal signaling molecule implicated in interspecies communication. AI-2 is detected by distinct receptor systems, such as the LuxP/LuxQ complex in Vibrio spp. and the Lsr transporter system in *Escherichia coli* and *Salmonella spp*. AI-2-mediated QS regulates biofilm formation, motility, and metabolic coordination in polymicrobial communities [46].

Escherichia coli lacks a canonical AHL synthase but possesses the SdiA receptor, which detects AHLs produced by other bacterial species. This enables *E. coli* to "eavesdrop" on neighboring microbial populations and modulate gene expression accordingly. Additionally, AI-2 signaling via the Lsr operon plays a crucial role in biofilm development and intestinal colonization [36].

QS systems are intimately linked with biofilm formation and maintenance. QS regulates the expression of adhesins, EPS biosynthetic enzymes, and dispersal factors. Spatial and metabolic heterogeneity within biofilms can create QS microenvironments, allowing subpopulations to respond differentially to the same signal. Moreover, QS interacts with secondary messengers such as cyclic-di-GMP, further fine-tuning the transition between planktonic and sessile lifestyles [26].

4. Biofilm Maturation

Biofilm maturation is the pivotal developmental stage during which an initially attached and proliferating microbial assemblage differentiates into a highly organized, three-dimensional, and functionally integrated community. This stage marks the transition from transient surface colonization to a stable, self-protective biological system with emergent properties that are absent in planktonic cells or immature biofilms . Maturation is driven by tightly regulated genetic programs, physicochemical gradients, and intercellular communication mechanisms that collectively enhance survival under hostile environmental and therapeutic pressures [13,14].

CAs maturation progresses, biofilms acquire complex architectures such as mushroom-like structures, ridges, and pillar formations interspersed with water channels. These channels are not passive voids but actively maintained conduits that facilitate convective transport of nutrients, oxygen, metabolites, and signaling molecules while allowing removal of toxic waste products. Architectural heterogeneity arises from localized differences in growth rates, matrix production, programmed cell death, and mechanical forces exerted by expanding extracellular polymeric substances (EPS), allowing the biofilm to function as a coordinated multicellular system rather than a uniform bacterial mass [43,21].

The extracellular polymeric substance undergoes profound qualitative and quantitative changes during maturation. Mature biofilms are dominated by a dense EPS matrix which plays critical structural role by cross-linking matrix polymers and stabilizing the biofilm, while matrix proteins facilitate cell–cell cohesion and surface attachment. This expanded matrix not only confers mechanical integrity but also acts as a selective permeability barrier, dramatically reducing the penetration and effective concentration of antimicrobial agents and host immune factors within deeper biofilm layers [23,27]. A hallmark of biofilm maturation is the emergence of pronounced physiological and metabolic heterogeneity. Steep gradients of oxygen, nutrients, pH, and redox potential develop across the biofilm depth, resulting in stratified microenvironments. Cells at the biofilm periphery typically exhibit higher metabolic activity, while cells in the interior adopt slow-growing or dormant states. This stratification supports functional specialization, where distinct subpopulations contribute differentially to matrix synthesis, stress resistance, or dispersal readiness. Such heterogeneity is a major determinant of biofilm resilience and ensures population survival during sudden environmental fluctuations or antimicrobial exposure [42,40].

During maturation, quorum sensing pathways frequently interact with intracellular second messengers such as cyclic di-GMP, which acts as a master regulator promoting sessility, matrix production, and repression of motility. Elevated cyclic di-GMP levels stabilize mature biofilm architecture and reinforce the sessile phenotype, locking cells into a biofilm-adapted state [26,44]. This also enhances genetic plasticity and evolutionary potential. The close spatial proximity of cells within the matrix facilitates horizontal gene transfer through transformation, conjugation, and bacteriophage-mediated transduction. Stress conditions prevalent in mature biofilms promote increased mutation rates and phenotypic variation, accelerating adaptive evolution. This genetic exchange and diversification contribute to the rapid dissemination of antimicrobial resistance determinants and virulence traits, particularly in chronic infections and long-term environmental biofilm habitats [30,16].

From a therapeutic standpoint, the maturation phase is the principal contributor to biofilm recalcitrance. Mature biofilms display tolerance to antimicrobials that can exceed planktonic susceptibility by several orders of magnitude. This tolerance arises from multiple overlapping mechanisms, including restricted drug diffusion, altered target expression, enzymatic degradation of antimicrobials within the matrix, reduced growth rates, and enrichment of persister cells. Persister cells, which are phenotypically tolerant rather than genetically resistant, are particularly abundant in mature biofilms and serve as a reservoir for infection relapse following cessation of therapy [29,18].

In host-associated biofilms, maturation further enables immune evasion. The EPS matrix masks pathogen-associated molecular patterns, impairs phagocytosis, and limits antibody and complement access. Mature biofilms often induce a chronic, low-grade inflammatory response that damages host tissues while failing to clear the infection, thereby reinforcing a stable niche for biofilm persistence. This immune modulation is especially relevant in chronic veterinary infections such as mastitis, wound infections, respiratory disease, and implant-associated infections [7,20].

In essence, biofilm maturation represents the culmination of biofilm development, conferring maximal structural stability, ecological fitness, and therapeutic tolerance. Once this stage is established, eradication becomes exceedingly difficult using conventional antimicrobial strategies alone [16]. A detailed understanding of biofilm maturation is therefore fundamental for designing effective anti-biofilm interventions, emphasizing the need for early disruption, matrix-targeted therapies, and strategies that interfere with regulatory networks sustaining the mature biofilm state.

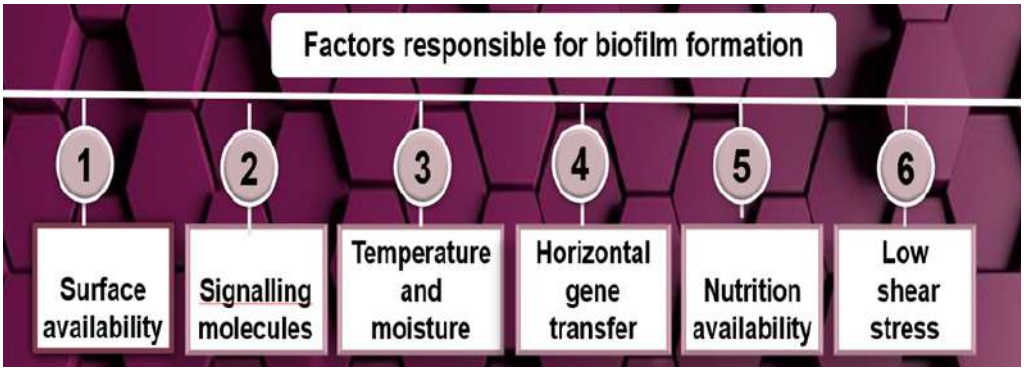

Figure 6: Environmental and biological factors influencing biofilm formation, including surface characteristics, nutrient availability, shear stress, and microbial communication.

5. Biofilm Dispersion

Dispersion represents the final stage of the biofilm life cycle and enables microbial dissemination to new sites. This process may occur passively through mechanical forces or actively through enzymatic degradation of the EPS matrix. Environmental cues such as nutrient depletion, accumulation of waste products, or changes in shear stress often trigger dispersion, releasing planktonic cells capable of initiating new biofilms [28].

Habitat of Biofilm

They are ubiquitous in nature and represent the predominant mode of microbial existence across diverse ecological niches. They are found in natural, engineered, and host-associated environments where surface availability, moisture, and nutrients support microbial attachment and persistence. The ability of microorganisms to form biofilms allows them to colonize stable habitats and survive under fluctuating environmental conditions that are often unfavorable for planktonic cells [12].

1. Natural Aquatic Environments

In aquatic ecosystems, biofilms commonly develop on submerged surfaces such as rocks, sediments, plant tissues, and aquatic organisms. These biofilms, often referred to as periphyton, play a crucial role in nutrient cycling, organic matter degradation, and primary productivity. The presence of flowing water promotes the formation of structurally robust biofilms adapted to shear stress, contributing significantly to microbial ecology in freshwater and marine habitats [6]. Soil environments provide highly heterogeneous surfaces for biofilm formation, including mineral particles, organic debris, and plant roots. This facilitate nutrient exchange, enhance microbial survival during desiccation, and support symbiotic interactions such as those occurring in the rhizosphere. Within soil aggregates, biofilms contribute to soil structure, stability, and biogeochemical cycling [36].

Biofilms frequently colonize plant surfaces, including phyllosphere, rhizosphere, and internal tissues. In the rhizosphere, biofilm-forming microorganisms interact closely with plant roots, influencing nutrient uptake, growth promotion, and disease suppression. These biofilms can be beneficial or pathogenic, depending on the microbial species involved and environmental conditions [14].

2. Industrial Food processing and agricultural environments

Biofilms are prevalent in food-processing facilities and agricultural settings, where they colonize surfaces such as stainless steel, plastic, rubber, and glass. Moist environments in dairy plants, slaughterhouses, and feed-processing units provide ideal conditions for biofilm development. Once established, these biofilms act as persistent sources of microbial contamination, compromising food safety and hygiene standards [46].

In engineered environments, biofilms form in water distribution systems, pipelines, cooling towers, and wastewater treatment plants. While some industrial biofilms cause biofouling and corrosion, others are harnessed for beneficial applications such as bioremediation and wastewater treatment. The adaptability of biofilms to engineered habitats highlights their ecological and industrial significance [22].

3. Biofilm in Hospitals

Hospitals represent highly favourable environments for biofilm formation due to constant moisture, frequent antimicrobial exposure, high surface availability, and the presence of immunocompromised patients. In such settings, biofilms serve as persistent microbial reservoirs that continuously seed pathogens into the hospital environment, contributing significantly to healthcare-associated infections. Unlike planktonic organisms, biofilm-associated microbes are difficult to eradicate and can survive routine cleaning and disinfection procedures, thereby sustaining long-term contamination [20].

In veterinary medicine, biofilm habitats are of particular importance because they underpin the persistence of chronic infections, therapeutic failure, and recurrent disease across multiple animal species and production systems [13,27]. Within animal hosts, biofilms preferentially establish on epithelial and mucosal surfaces exposed to moisture, nutrients, and mechanical stress. The skin, respiratory mucosa, gastrointestinal epithelium, and secretory tissues provide ideal conditions for initial microbial attachment followed by biofilm maturation. In chronic dermatological infections of companion and food animals, biofilms formed by staphylococci and streptococci become deeply embedded within keratin layers and follicular structures, creating protected niches that are poorly accessible to topical antimicrobials and host immune cells [32,34]. These biofilm habitats are characterized by gradients of oxygen, pH, and nutrients, which promote phenotypic diversification and metabolic dormancy, enabling long-term persistence under antimicrobial pressure. In bovine mastitis, biofilms formed by *Staphylococcus aureus, Streptococcus uberis*, and coagulase-negative *Staphylococci* adhere to mammary epithelium, milk fat globules, and ductal surfaces, resulting in chronic and subclinical infections that are refractory to conventional intramammary therapy [38,41]. Biofilm growth within the mammary gland alters bacterial gene expression, reduces metabolic activity, and promotes the survival of persister cells, thereby explaining the frequent discrepancy between in-vitro antimicrobial susceptibility results and poor *in-vivo* cure rates [31,5].

Respiratory tract biofilms further illustrate the adaptive capacity of bacterial communities within hosts. In livestock species, pathogens associated with respiratory disease complexes colonize nasal passages, tracheal epithelium, and pulmonary tissue, where biofilms interact with mucus, inflammatory exudates, and necrotic debris. These habitats generate localized hypoxic and nutrient-limited conditions that favor slow-growing or dormant bacterial subpopulations, reducing the effectiveness of antimicrobials targeting cell wall synthesis or protein production [20,9]. In chronic pneumonia, particularly in cattle, biofilm-associated pathogens persist despite aggressive metaphylactic treatment, contributing to relapse and long-term productivity losses. The gastrointestinal tract represents a highly complex biofilm habitat where commensal and pathogenic microbial communities coexist in close association with mucosal surfaces and digesta. While commensal biofilms play a critical role in maintaining gut homeostasis, pathogenic biofilms formed by enteric bacteria such as *Salmonella, Escherichia coli*, and *Clostridioides perfringens* enable prolonged colonization and intermittent shedding without overt clinical disease [19,8].

Abiotic biofilm habitats are equally significant in medical devices such as urinary catheters, orthopedic implants, sutures, and endotracheal tubes provide non-shedding surfaces that favor microbial adhesion and biofilm development. Once established, these biofilms are highly tolerant to systemic antimicrobials due to limited drug penetration and reduced immune surveillance at the implant interface [8,22]. In surgery and intensive care, device-associated biofilms frequently necessitate implant removal to achieve clinical resolution, highlighting the limitations of antimicrobial therapy alone.

Beyond the individual animal, environmental biofilm habitats in farms and veterinary facilities exert a critical influence on disease persistence and therapeutic outcomes. Biofilms in water distribution systems, feeding equipment, milking machinery, and housing surfaces protect pathogens from desiccation, disinfectants, and temperature fluctuations, allowing long-term survival and repeated exposure of animals to infectious agents[9] [38]. These environmental reservoirs undermine individual animal treatment and emphasize the need for integrated biofilm control strategies at the herd and farm levels.

From a therapeutic perspective, biofilm habitats fundamentally alter antimicrobial efficacy through multiple, overlapping mechanisms, including restricted diffusion of drugs through the extracellular polymeric matrix, altered local microenvironmental conditions, reduced bacterial growth rates, and the presence of persister cells with transient tolerance to antimicrobial agents [39,30]. These factors collectively explain why biofilm-associated infections in veterinary medicine often require prolonged treatment, combination therapy, or non-antibiotic interventions to achieve partial or complete resolution. A comprehensive understanding of biofilm habitats is therefore essential for advancing evidence-based veterinary therapy, improving clinical outcomes, and reducing reliance on antimicrobials in animal health systems.

1. Enhanced Antimicrobial Tolerance and Treatment Failure

One of the most critical clinical implications of biofilms in hospitals is their remarkable tolerance to antimicrobial agents. Biofilm-embedded microorganisms may require antimicrobial concentrations up to 1000 times higher than those effective against planktonic cells. This tolerance arises from restricted drug penetration, reduced metabolic activity of biofilm cells, activation of efflux pumps, and the presence of persister cells. Consequently, conventional antibiotic therapy often fails, leading to prolonged hospitalization and repeated treatment cycles [25].

Hospital biofilms act as hotspots for the development and dissemination of antimicrobial resistance. The dense and stable nature of biofilms promotes horizontal gene transfer through plasmids, transposons, and extracellular DNA. This genetic exchange facilitates the rapid spread of resistance determinants among nosocomial pathogens, contributing to the emergence of multidrug-resistant organisms such as MRSA, VRE, and carbapenem-resistant Gram-negative bacteria [31]. Beyond medical devices, biofilms colonize hospital infrastructure, including sinks, drains, faucets, showerheads, water distribution systems, air-conditioning units, and humidifiers. These environmental biofilms are recognized sources of opportunistic pathogens such as Pseudomonas aeruginosa, Acinetobacter baumannii, Legionella pneumophila, and non-tuberculous mycobacteria. Such reservoirs contribute to indirect transmission through aerosols, water contact, and contaminated surfaces, complicating infection control efforts [2].

Biofilms pose a major challenge to hospital sanitation practices, as standard disinfectants are often ineffective against mature biofilms. The extracellular matrix limits disinfectant penetration and can neutralize biocidal agents, allowing microbial survival even after rigorous cleaning. This necessitates the development of enhanced infection control strategies, including anti-biofilm surface coatings, enzymatic cleaners, and novel disinfection technologies [26].

2. Economic and Public Health Impact

Biofilm-associated hospital infections impose a substantial economic burden due to prolonged hospital stays, increased diagnostic procedures, repeated antimicrobial therapy, and additional surgical interventions.

From a public health perspective, the persistence of biofilms in hospitals contributes to increased antimicrobial usage and the global spread of resistant pathogens. Addressing biofilms is therefore essential not only for patient safety but also for sustainable healthcare management [47].

Importance of Biofilm in Antimicrobial Resistance

Biofilms play a central role in the emergence, persistence, and dissemination of antimicrobial resistance by providing a protective ecological niche that favors microbial survival under antimicrobial pressure [41]. Within biofilms, microorganisms exhibit markedly reduced susceptibility to antibiotics compared to planktonic cells, contributing significantly to treatment failure in both clinical and veterinary settings. This biofilm-associated tolerance is distinct from classical genetic resistance and represents a major challenge in antimicrobial chemotherapy [20].

1. Physical Barrier to Antimicrobial Penetration

The extracellular polymeric substance (EPS) matrix of biofilms acts as a physical and chemical barrier that limits the diffusion of antimicrobial agents. Polysaccharides, proteins, extracellular DNA, and lipids within the matrix can bind, neutralize, or delay antibiotic penetration, resulting in sub-inhibitory drug concentrations in deeper biofilm layers. This incomplete penetration allows microbial cells to survive exposure to otherwise lethal antimicrobial doses [39].

2. Altered Physiological States of Biofilm Cells

Microorganisms within biofilms exist in heterogeneous physiological states due to nutrient, oxygen, and pH gradients. Cells located in the deeper layers often exhibit reduced metabolic activity and slower growth rates, rendering them less susceptible to antimicrobials that target active cellular processes. This metabolic dormancy significantly contributes to the phenotypic tolerance observed in biofilm-associated populations [42]. Biofilms harbor specialized subpopulations known as persister cells, which are transiently tolerant to high concentrations of antimicrobials without possessing heritable resistance traits. These cells survive antibiotic treatment and can repopulate the biofilm once therapy is discontinued, leading to recurrent and chronic infections. The presence of persister cells represents a critical mechanism linking biofilm formation to antimicrobial treatment failure [29].

3. Enhanced Expression of Resistance Mechanisms

Biofilm growth induces the upregulation of multiple resistance-associated genes, including those encoding efflux pumps, stress response proteins, and antibiotic-modifying enzymes. These adaptive responses are often regulated by global stress regulators and quorum sensing systems, further increasing antimicrobial tolerance. Such coordinated gene expression enhances survival under prolonged antimicrobial exposure [31].

4. Horizontal Gene Transfer within Biofilms

Biofilms provide an optimal environment for horizontal gene transfer due to high cell density, close cell-to-cell contact, and the presence of extracellular DNA. Conjugation, transformation, and transduction occur at higher frequencies within biofilms, facilitating the rapid dissemination of antimicrobial resistance genes among microbial populations. This contributes to the emergence of multidrug-resistant strains in clinical and environmental settings [3].

5. Sub-Inhibitory Antimicrobial Exposure

In biofilm environments, microorganisms are often exposed to sub-inhibitory concentrations of antimicrobials due to limited penetration and uneven distribution. Such exposure does not eradicate biofilms but instead promotes stress adaptation, biofilm maturation, and selection of resistant phenotypes. Sub-therapeutic antimicrobial exposure within biofilms is therefore a key driver of antimicrobial resistance evolution [3].

Biofilm-mediated antimicrobial resistance significantly impacts clinical outcomes by prolonging infections, increasing relapse rates, and necessitating higher or prolonged antimicrobial dosing. From a public health perspective, biofilms act as persistent reservoirs of resistant pathogens in hospitals, veterinary facilities, and food-processing environments, accelerating the global antimicrobial resistance crisis [25].

Recognition of biofilm habitats has driven the development of novel therapeutic strategies aimed at disrupting biofilm structure and function rather than solely targeting bacterial viability. Approaches such as enzymatic degradation of matrix components, quorum sensing inhibition, phage therapy, and nanotechnology-based drug delivery systems are increasingly explored in veterinary contexts to overcome the limitations of conventional antimicrobial therapy [37,1].

Methods for Detection of Biofilm

Detection and characterization of biofilms are essential for understanding microbial pathogenicity, antimicrobial resistance, and persistence in clinical, veterinary, food, and industrial environments as biofilms exhibit structural complexity and physiological heterogeneity, no single method is sufficient for comprehensive detection.

Therefore, a combination of phenotypic, microscopic, biochemical, and molecular approaches is commonly employed to assess biofilm formation, biomass, viability, and architecture [18].

1. Phenotypic and Screening Methods

Phenotypic screening methods are widely used as initial tools for identifying biofilm-producing microorganisms due to their simplicity and cost-effectiveness. These assays primarily evaluate surface adherence and extracellular matrix production under laboratory conditions. Although they do not replicate in-vivo environments completely, they provide valuable baseline information for comparing biofilm-forming potential among isolates [42].

1.1. Tissue Culture Plate Assay (Microtiter Plate)

The tissue culture plate (TCP) assay is regarded as the gold standard for quantitative in-vitro detection of biofilm formation. Microbial suspensions are incubated in polystyrene microplates, allowing adherence and biofilm development, followed by staining with crystal violet. The bound dye is solubilized and measured spectrophotometrically, providing quantitative estimation of total biofilm biomass. This method is highly reproducible, scalable, and suitable for antimicrobial and anti-biofilm screening studies [34].

1.2. Tube Adherence Method

The tube adherence method is a qualitative assay used to visually detect biofilm formation on glass or plastic surfaces. After incubation, tubes are stained with crystal violet and examined for visible biofilm lining the walls. Despite its subjective nature and lower sensitivity compared to microtiter assays, this method remains useful for rapid screening and teaching laboratories where resources are limited [11].

1.3. Congo Red Agar (CRA) Method

The Congo red agar method detects biofilm formation based on the production of exopolysaccharides that bind Congo red dye. Biofilm-producing strains form black, dry, crystalline colonies, whereas non-producers produce smooth red colonies. This method is particularly useful for detecting slime production in Staphylococcus species but may yield variable results due to medium composition and incubation conditions [24].

2. Microscopic Techniques

Microscopy-based methods provide direct visualization of biofilm morphology and spatial organization. Light microscopy allows basic observation of stained biofilms, while scanning electron microscopy (SEM) offers detailed surface imaging of microbial cells and matrix components. Transmission electron microscopy (TEM) further reveals ultrastructural details. However, these methods often require extensive sample preparation that may disrupt biofilm structure [26].

Confocal Laser Scanning Microscopy (CLSM) is a powerful tool for three-dimensional visualization of biofilms in their hydrated state. Using fluorescent dyes and optical sectioning, CLSM enables detailed analysis of biofilm thickness, architecture, and cell viability. This technique allows real-time observation of live biofilms and is widely used for studying biofilm development, antimicrobial penetration, and spatial heterogeneity [29].

3. Fluorescence and Viability-Based Staining

Fluorescent staining techniques are employed to differentiate live and dead cells within biofilms and to identify matrix components. Dyes such as SYTO 9 and propidium iodide are used in viability assays, while lectin-based stains target polysaccharides in the EPS matrix. These methods provide functional insights when combined with CLSM or epifluorescence microscopy [33].

4. Biochemical and Metabolic Activity Assays

Biochemical assays evaluate biofilm viability by measuring metabolic activity rather than biomass alone. The XTT reduction assay, resazurin (Alamar Blue) assay, and tetrazolium salt assays quantify cellular respiration within biofilms. These methods are particularly valuable for assessing antimicrobial efficacy against biofilm-embedded microorganisms and distinguishing between live but non-growing cells and dead cells [35].

5. Molecular Detection Methods

Molecular techniques provide insight into the genetic basis of biofilm formation. Polymerase chain reaction (PCR) and quantitative PCR (qPCR) are used to detect biofilm-associated genes encoding adhesins, EPS components, and quorum-sensing regulators [1]. Gene expression studies using RT-qPCR and transcriptomics reveal differential regulation of genes during biofilm growth compared to planktonic states [15].

6. Advanced and Emerging Detection Techniques

Recent advances in biofilm detection include atomic force microscopy (AFM), biosensors, microfluidic platforms, and optical coherence tomography (OCT). AFM allows nanoscale measurement of biofilm surface properties and adhesion forces, while microfluidic systems enable real-time monitoring of biofilm development under controlled flow conditions. These techniques provide high-resolution, dynamic insights into biofilm behaviour and antimicrobial interactions [21].

Conclusion

Biofilms represent a highly adaptive microbial lifestyle that promotes persistence, immune evasion, and antimicrobial tolerance. Their structural complexity and regulatory networks contribute to chronic infections, environmental contamination, and treatment failure across medical and veterinary systems. Future biofilm control strategies should focus on disrupting matrix integrity, quorum sensing, and microbial communication rather than solely targeting bacterial viability. Advancing biofilm-aware therapeutics and antimicrobial stewardship is essential for mitigating antimicrobial resistance within the One Health framework.

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