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Biofilms: structure, resistance mechanism, emerging control strategies, and applications

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Biofilms are biological barriers produced by a variety of organisms either for defense or because of physiological processes. Many microorganisms produce biofilms to adapt to certain adverse conditions and this has resulted in difficulty in their eradication with antimicrobial agents. There is the increasing menace of antimicrobial resistance (AMR) by bacteria due to the production of biofilms. Specifically, bacterial biofilms are complex surface-delimited microbial structures contained in a matrix of extracellular polymeric substances, which are an obstacle to effective medical treatment of infections caused by these bacteria. Biofilm resistance to antibiotics can lead to persistent infections. Of particular concern are biofilms made from ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*), which are bacteria resistant to the action of many antimicrobial agents. Following the emergence of AMR attributed to biofilms, which complicates disease treatment options and increases morbidity and mortality, there is an urgent need to understand the underlying mechanism of the formation of biofilms, their structure and the resistance profiles, strategies, and barrier systems, which have not been sufficiently considered all together. This systematic review can enable the precipitation of findings and control strategies for the development of effective interventions, guide research efforts, and inform clinical practices in handling biofilms. This review focuses on the different characteristics of biofilms, the organization of biofilms, the life cycles, and various models for studying biofilms, as well as the ways through which biofilms can be resistant to antimicrobials. The strategies for biofilm management, the role played by biofilms in clinical practice, and promising paradigms for the assessment of the outcome will also be highlighted. With the knowledge of how biofilms function and their relation to pathogens, life scientists can more effectively develop management plans to eradicate biofilm-related infections and provide better patient care.

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1 Introduction

Biofilms are considered one of the most captivating and challenging realizations of microbial life on our planet. They are described as complex organized microbial societies contained in a matrix of extracellular polymeric substances (EPS) (composed of nucleic acids, polysaccharides, and proteins) and can develop and exist on natural abiotic and biotic substrates as well as on artificial materials and medical instruments.[1,2] This complex structure enables small microbes to exist in environments that would be quite unsuitable for a planktonic cell.[3] In environmental contexts, biofilms are of importance for nutrient cycling in ecosystems; in clinical contexts, biofilms are an obstacle to effective medical treatment of infections.[4,5]

Biofilms are aggregates of microbial cells that are embedded in a complex extracellular polymeric matrix (Fig. 1) that develops through several stages involving bacterial attachment to a surface, microbial growth, maturation, and dispersion.[6] This process is affected by many factors in its environment, for instance, the available nutrient concentrations, microbial species present, flow, and the nature of the substrate surface. Biofilms that form are permanent structures with defined architectural features that provide the basis for physical protection of the bacteria within the biofilm from

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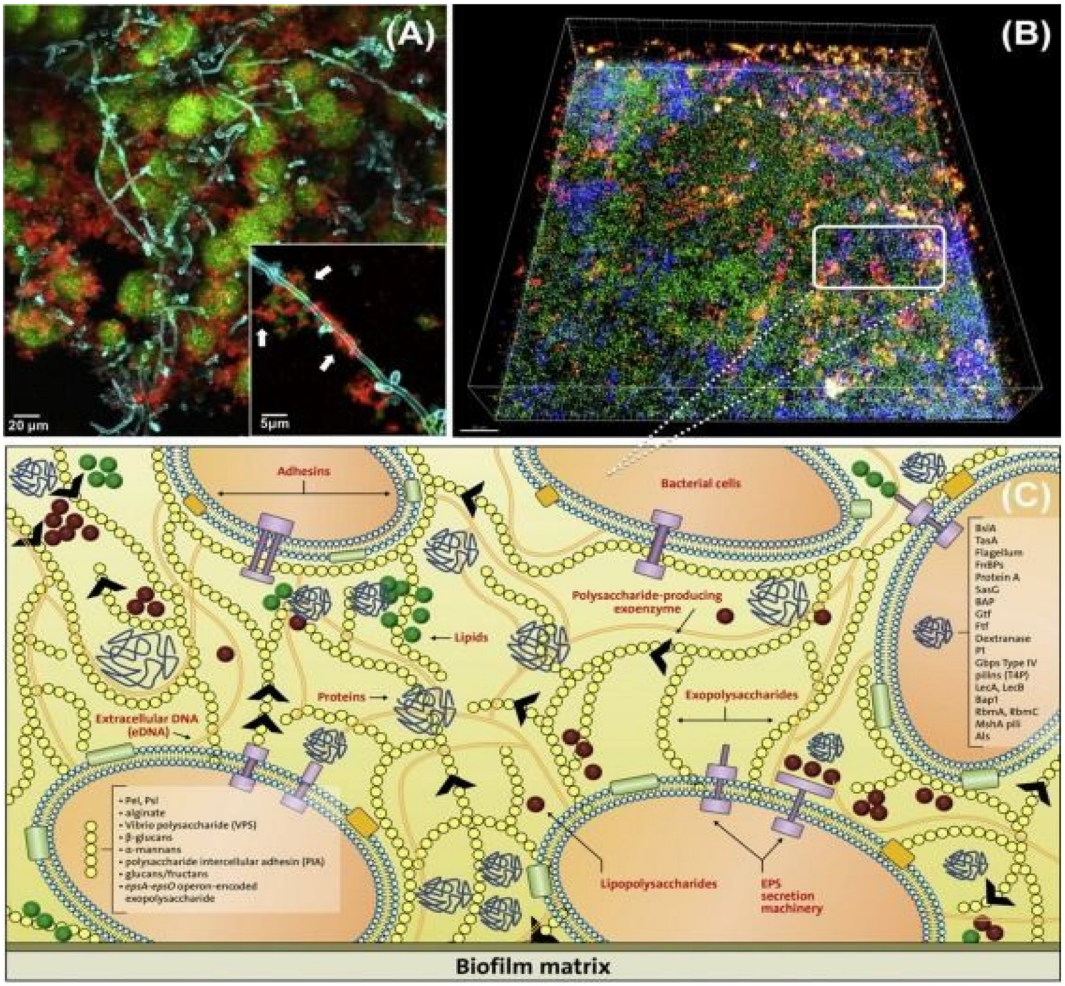

Fig. 1 The complex extracellular polymeric matrix of biofilms showing their components. Reproduced from ref. 8 with permission from Cell Press (Elsevier), Cambridge, MA, USA, copyright 2020.

physical, chemical and biological attack.[3,7] These structures may asymmetrically hold bacteria, fungi and sometimes protozoa, which interdependently exist, and improve their survival as well as functionality.

With favourable conditions, biofilms initially adhere reversibly to suitable surfaces, through the formation of weak interactions including electrostatic interactions and van der Waals forces.[9,10] They, however, become strongly attached (sessile) through the secretion of the sticky three-dimensional EPS matrix.[1,2] Architecturally, distinct microcolonies are formed within the biofilms with different compositions and sizes, thus creating a heterogeneous and diverse environment which allows the effective exploitation of niches.[2,11] Specifically, while some of the microbial communities within the biofilms thrive in nutrient- or oxygen-rich environments, others thrive otherwise, with such spatial organization generating gradients of nutrient utilization and waste products which influence microbial interactions and behaviour.[12,13] Importantly, the different microbial communities of a biofilm engage in sophisticated communications through quorum sensing, allowing for effective coordination and adaptation to environmental changes, including resistance to threats including antimicrobial agents.[3]

The control measures against biofilms are multifaceted and have significant implications across various areas. The traditional control measures, including the use of currently available antibiotics, are increasingly becoming ineffective.[14,15] Biofilm resistance to antibiotics can lead to persistent infections, and equipment corrosion and contamination. The control strategies for biofilms involve the use of antimicrobial coatings and surfaces which prevent the formation of biofilms.[15] There are also disruptive techniques, including mechanical removal and dislodging of biofilms using high-pressure water jets,[16] the use of chemical agents such as enzymes and surfactants that weaken/compromise EPS, making it susceptible to antimicrobial agents,[17,18] the use of ultrasound and other physical methods which create cavitation bubbles which disrupt biofilms' integrity,[19,20] the use of biological control techniques, such as the use of bacteriophages to target specific microbial species and certain other beneficial bacteria through competitive exclusion or production of inhibitory compounds that can inhibit the growth of biofilm-

forming species,[21,22] and the use of antimicrobial treatments, either in combination therapies or through the discovery, development, and application of new classes of antimicrobial agents, including peptides and nanomaterials.[23,24]

Amidst the effort against disease-implicated biofilms, they usually devise resistance mechanisms, which are of public health importance. Some of the resistance mechanisms involve the acquisition of resistance genes, through gene transfer, heterogeneity of metabolic activities (physiological changes) which can impact dormancy, phenotypic resistance such as the secretion of protective enzymes and the impeding of antimicrobial agents through the modification of the surface properties.[25,26] Of particular concern are biofilms made from ESKAPE pathogens, which are bacteria resistant to the action of antimicrobial agents. ESKAPE stands for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, all of which give rise to healthcare-associated diseases.[27,28] The biofilm architecture of these pathogens can develop on medical devices and tissues, which creates a major challenge in treatment. The relation between biofilms and antimicrobial resistance (AMR) responses has also become an active research concern, illustrating the need for adequate anti-biofilm measures.[29]

Following the emergence of AMR attributed to biofilms, which complicates disease treatment options and increases morbidity and mortality, there is an urgent need to understand the underlying mechanism of the formation of biofilms, their structure and the resistance profiles, strategies, and barrier systems, for which the ongoing experimentation and reports have not been sufficiently considered all together. Such review would enable the precipitation of findings and control strategies for the development of effective interventions, guide research efforts, and inform clinical practices. Thus, this current review is aimed at focusing on the different characteristics of biofilms, the organization of biofilms, their life cycles, antimicrobial resistance mechanisms, emerging management options, and various models for studying biofilms. Also, the strategies for biofilm management, the role played by biofilms in clinical practice, and promising paradigms for the assessment of the outcome are discussed. With knowledge of how biofilms function and their relation to pathogens, we can more effectively develop management plans to eradicate biofilm-related infections and provide better patient care.

2 Biofilm architecture and development: structural dynamics and lifecycle

2.1 Stages and pathways of biofilm development

Biofilm formation is a multifaceted process involving physical, chemical, and biological elements. It typically unfolds in several stages, beginning with the initial reversible attachment of free-floating microorganisms to surfaces that have been preconditioned. This stage is succeeded by a shift to irreversible attachment, which is initiated by the production of extracellular polymeric substances.[30] Following this, microcolonies evolve into mature biofilms, which are then released into the surrounding environment, allowing the cycle to start anew.[31] Bacteria within biofilms exhibit significantly different traits compared with their planktonic counterparts, including variations in physiology and increased resistance to the host's immune defenses and antimicrobial treatments.[30,31] Studies indicate that these phenotypic changes result from alterations in the infection microenvironment, such as shifts in nutrient availability, temperature, pH, and ionic strength. To devise effective strategies for managing biofilm-associated infections, it is crucial to comprehend the biofilm's structure, formation, composition, and properties. This understanding is also essential for leveraging beneficial biofilms in industrial and environmental applications.

2.1.1 Initial reversible attachment. Microorganisms have the ability to attach to a lot of different surfaces. In this step of biofilm formation, single cells of microorganisms adhere to precondition surfaces (surfaces with enough moisture and nutrients to support microbial propagation). The nature of the surface plays a vital role in enabling or preventing the adhesion of microorganisms. For instance, the physical properties of a surface such as roughness have been reported to affect the adhesion of microbial cells.[31] Studies have reported that rough surfaces tend to promote better initial microbial adhesion and propagation compared with smooth surfaces.[32,33] In some bacteria such as *Pseudomonas fluorescens*, the process of adhesion to surfaces occurs *via* a passive process and involves the use of structures such as pili, (usually 5–25 nm wide and 1–2 μm long).[33] In other cases, the microorganisms employ a more active approach to adhesion. This strategy, however, requires prolonged exposure to the surfaces in other to firmly attach. Research has also shown that the same bacterial species may employ either a passive or active approach to attach to different surfaces.[31] Usually, this initial attachment is reversible because the bacteria usually attach to the surface *via* weak interactions, such as van der Waals forces and electrostatic interactions.[33,34]

2.1.2 Irreversible attachment and maturation. At this stage the reversibly attached cells utilize the nutrients in the microenvironment to grow and divide, developing the characteristic 3-D structure of the biofilm.[30,35] In contrast to the initial attachment stage where physical and chemical processes are pivotal factors, biological processes such as the production of polysaccharide intercellular adhesion (PIA) polymer dominate this stage of attachment.[35] This step is usually initiated by the production of EPS, which brings about a stronger level of interaction between the adhered microbial cells and the surface. In addition to providing structural stability in the biofilm, the EPS also protects the bacterial community from harmful agents and environments such as antibiotics and host immune systems.[31] This production of the EPS signals the commencement of the maturation process as well as quorum-sensing initiation.[34,35] At very high cell density, the microcolonies in the biofilm develop various mechanisms for cell signaling known as quorum sensing. This mediates communication within the bacterial species as well as between other microbial

species. Although quorum sensing systems usually involve the production, detection, and response to extracellular signaling molecules called autoinducers, they differ in various bacteria species.[33] While the autoinducers for Gram-positive bacteria include specific peptides, those for Gram-negative are primarily composed of acylated homoserine lactones and are important for the regulation of the bacterial population.[30,31,36,37]

2.1.3 Dispersal. This represents the final stage in the biofilm formation. Here the attached cells from the mature biofilms detach and move to colonize a new environment, thus initiating the spread of the bacterial population as well as the formation of new biofilms. Cells from an existing biofilm can either be dispersed by the shedding of cells from other growing cells or the detachment can be caused by limited nutrition in the microenvironment, fluid dynamics and the effect of secretory proteins (Garg *et al.*, 2023) (Fig. 2).

2.2 The key biofilm components and their roles in resistance and persistence

Biofilms are highly complex systems and are composed of different constituents which perform unique functions in the biofilm, contributing to the intricacy of this system. These components work synergistically to create a resilient structure that enables biofilms to survive in harsh environments, resist antimicrobial treatments, and persist in both *in vivo* and *in vitro* conditions. The key biofilm components include microorganisms, extracellular polymeric substance (EPS), water channels, and nutrients (Fig. 3).

The primary component of biofilms is the microorganisms, which vary widely depending on the environment in which the biofilm forms.[39,40] Bacteria, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*, are commonly found in biofilms associated with medical infections.[38,41] The EPS matrix is another essential component of biofilms. This matrix, comprising polysaccharides, proteins, lipids, enzymes and extracellular DNA (eDNA), provides structural integrity to the biofilm and shields the microbial community from harmful substances and environments including antibiotics and different host immune responses. The EPS also serves as a protective barrier against desiccation and contributes immensely to the persistence of biofilms. Additionally, the EPS helps trap nutrients and creates a microenvironment that promotes microbial growth and stability.[35,36,38] Polysaccharides are widely recognized for providing the mechanical support necessary for biofilms and are believed to constitute a significant portion of the exopolymeric substance. Among these, alginates, a class of natural polysaccharides produced by bacterial species like *Pseudomonas* and *Azotobacter*, play a vital role.[42–44] Alginates primarily function to protect bacteria from environmental stresses, offering mechanical reinforcement, particularly under conditions where water is scarce. In addition to polysaccharides, bacteria within biofilms secrete various proteins that contribute to biofilm stability. These include carbohydrate-binding proteins, often referred to as lectins or glycoproteins, which are essential for the formation and stabilization of the biofilm matrix. Extracellular DNA (eDNA) also plays a crucial role in biofilm development. Released through autolysis, eDNA is found in various bacterial species such as *Streptococcus*, *Staphylococcus*, *Enterococcus*, and notably *Pseudomonas aeruginosa.*[33] eDNA serves to protect bacteria from antimicrobial agents and immune responses by binding to and stabilizing bacterial membranes, as well as by chelating

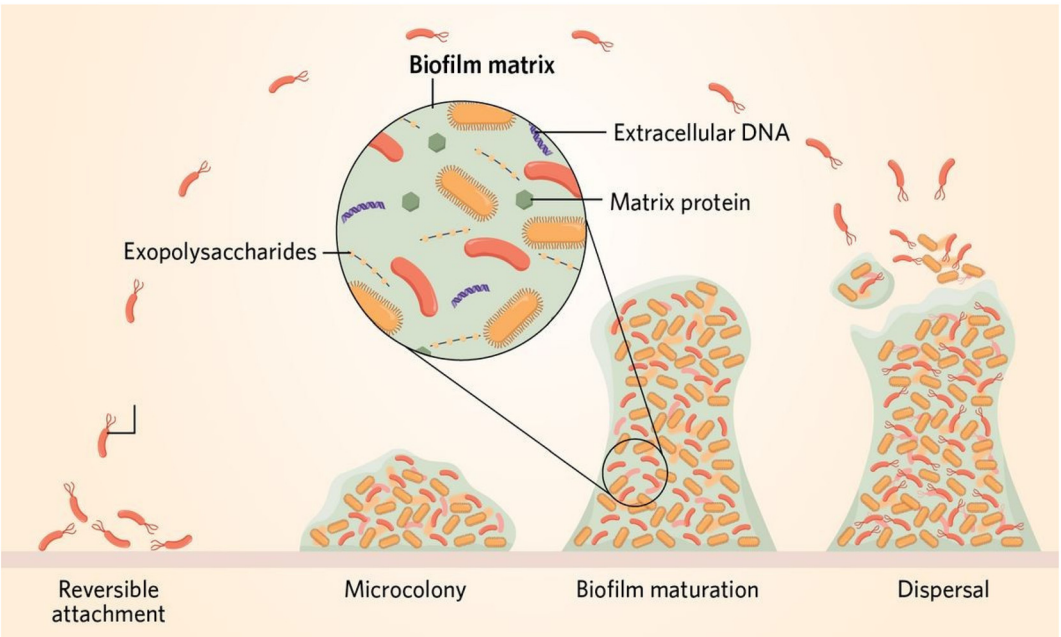

Fig. 2 Different stages of biofilm formation indicating the reversible attachment, microcolony, biofilm maturation and dispersal stages. Reproduced from ref. 33 with permission from Frontiers Media SA, Lausanne, Switzerland, copyright 2023.

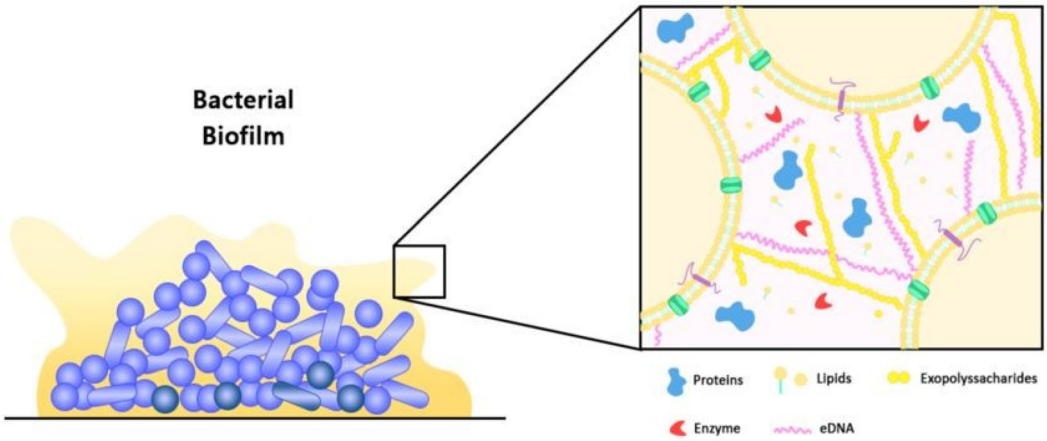

Fig. 3 EPS structure of bacterial biofilms with their components (proteins, lipids, exopolysaccharides, enzymes and eDNA). Reproduced from ref. 38 with permission from Elsevier B.V., Amsterdam, The Netherlands, copyright 2023.

cations that promote cell lysis. Another important element of the EPS is biosurfactants, which assist bacteria in attaching to and detaching from surfaces, such as oil droplets, aiding in biofilm formation and dispersal.[35] The biofilm microenvironment also has numerous water channels. These channels allow the efficient transport of nutrients, oxygen, and signaling molecules to the cells deep within the biofilm. They also facilitate the removal of waste products. The organization of these channels helps maintain a balance between the core and surface layers of the biofilm, ensuring that even the microorganisms located deep within the biofilm receive adequate resources to survive. Nutrients are critical for biofilm development and sustainability. Biofilms are typically found in nutrient-rich environments, such as medical devices, wounds, or industrial water systems. Microorganisms within the biofilm can metabolize these nutrients and, through their metabolic activities, alter the chemical composition of their surroundings. This metabolic diversity allows biofilms to thrive in a wide range of environments, from highly oxygenated surfaces to anaerobic conditions deep within the biofilm layers.[45]

2.3 Biofilms and their unique physiological properties: implications for pathogenicity and treatment failure

Biofilms are very heterogeneous. Studies have identified different microbial populations in different biofilm regions and these microorganisms usually possess unique phenotypic properties.[45–47] This type of variation among bacterial cells is as a result of factors such as genetic differences and epigenetic modification as well as environmental factors. This heterogeneity is responsible for some bacteria being metabolically active, especially those on the biofilm surface, while others, for instance those in the deeper parts of the biofilm, remain more dormant.[46] This variation impedes the complete eradication of the bacteria population as only the metabolically active bacteria on the biofilm surface are killed by antimicrobial agents, leaving the less active cells within the biofilm; these subsequently cause a reinfection. Also, the metabolic state of microbial cells in the biofilm varies remarkably from planktonic cells; this is because the biofilm is usually hypoxic, and as such most of the microorganisms are metabolically inactive. Usually, during the lag phase, the oxygen consumption occurs rapidly, and when the oxygen level reduces, the microbial growth rate also reduces.[46,48,49] Also, the presence of other oxygen consumers especially *in vivo* enables a persistent hypoxic condition surrounding the biofilms. The drastic reduction in metabolic rate is a key strategy in the development of resistance to antimicrobial agents. This is because the reduced rate of growth is also responsible for rendering antibiotic targets such as protein synthesis inactive. In addition, biofilms can also limit the penetration of antimicrobial agents, thereby protecting bacterial cells from being killed by these agents. Furthermore, the biofilm also enhances the expression of certain genes that bring about transcriptional tolerance. For instance, studies report that an increased expression of c-di-GMP within the microorganisms in the biofilm upregulates efflux pumps, a key strategy for resistance in many microorganisms.[33,46]

Overall, the properties of biofilms—protection, resource efficiency, horizontal gene transfer, and phenotypic diversity—contribute to their persistence and resilience in a wide range of environments. These characteristics make biofilms particularly problematic in clinical and industrial contexts, where they pose significant challenges to treatment and control. Therefore, a clear understanding of the formation, components and unique properties of biofilms is vital in the diagnosis, treatment and prevention of clinical infections (Fig. 4).

3 Mechanisms of antimicrobial resistance in biofilm-forming microorganisms

3.1 Bacterial pathogens with biofilm-mediated resistance

i. *Listeria monocytogenes*

L. monocytogenes, a saprophytic bacterium, is a Gram-positive pathogen found in soil, water, food and food products

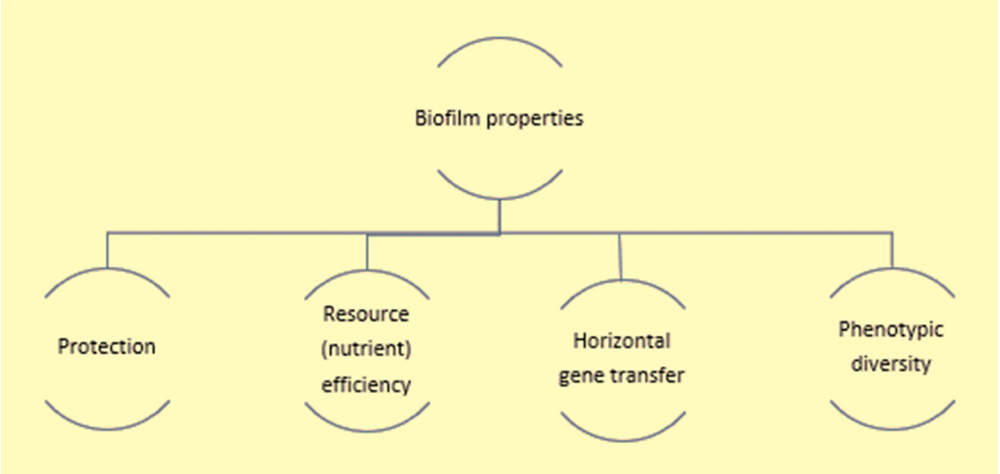

Fig. 4 Properties of biofilms responsible for their persistence and resilience to antimicrobials. Reproduced from ref. 46 with permission from Oxford University Press, Oxford, United Kingdom, copyright 2023.

such as dairy products, meat, fish, shrimp, shellfish *etc*., causing listeriosis, particularly in vulnerable individuals.[50–52] Its ability to form biofilm enables persistence in food processing environments.[53]

ii. *Salmonella* spp.

A Gram-negative bacterium present in many animals, *Salmonella* spp. are transmitted through water and food, moving across the food chain to humans susceptible to its infection.[54,55] *Salmonella* spp. cause Salmonella poisoning and are a major challenge for animal breeders and the food industry. The pathogen is a leading cause of human diarrhoeal diseases worldwide, frequently associated with poultry, eggs, pigs, cattle, and fresh produce.[56,57] *Salmonella* biofilms on equipment surfaces can be a continuous source of contamination.

iii. *Escherichia coli* (*E. coli*)

E. coli is one of the species most frequently involved in biofilm-related diseases, being especially important in urinary tract infections, causing relapses or chronic infections. These bacteria can cause severe and potentially life-threatening illnesses, including hemorrhagic colitis, hemolytic uremic syndrome, and acute kidney failure. *E. coli*'s ability to form biofilms poses significant challenges for public health. Its ability to form biofilms confers protection from antibiotic treatment and the immune system.[58–60]

iv. *Pseudomonas* spp.

A virulent rod-shaped, Gram-negative bacterium from the Pseudomonadaceae group, found in water, plants, soil, and animals, *Pseudomonas* spp. rarely cause infections in healthy individuals but easily infect immune-compromised ones.[61] It is a major biofilm-forming pathogen in food products, causing food spoilage. *P.* spp. include *P. fluorescens*, *P. putida*, *P. brenneri*, *P. koreensis*, and *P. aeruginosa.*[62–66]

P. aeruginosa is involved in persistent biofilm infections, including cystic fibrosis (CF) lung infections, chronic wound infections, urinary tract infections with or without catheters, and tracheal tube-related ventilator-associated pneumonia.[67]

v. *Vibrio parahaemolyticus*

A curved, Gram-negative bacterium present in marine environments, *Vibrio parahaemolyticus* forms biofilms on marine biotic and abiotic surfaces under appropriate conditions, functioning as a source of pathogenic bacteria with 10–1000 times the resistance to hygiene treatments than its planktonic counterparts.[45] As one of the most common foodborne pathogenic bacteria that forms biofilms, it contaminates seafood products, with infections typically linked to raw or undercooked seafood consumption.[68,69]

vi. *Aeromonas hydrophila*

Aeromonas hydrophila is a bacterium, widely distributed in aquatic environments, which can cause a wide range of infectious diseases in aquatic animals. The most encountered *Aeromonas* species can infect fish, amphibians, and humans. It is frequently isolated from both unprocessed and processed seafood products, causing illnesses with symptoms similar to those caused by *Vibrio parahaemolyticus.*[70,71] It engages in a variety of human illnesses and can easily adhere to surfaces.[72]

vii. *Staphylococcus aureus*

A Gram-positive pathogenic bacterium and a major cause of different infectious illnesses in humans and animals, *Staphylococcus aureus*-related infections are problematic and are difficult to treat due to biofilm formation.[73] It is also capable of producing enterotoxins, and can form biofilms on equipment surfaces, posing a significant risk of foodborne illness.[74]

viii. *Acinetobacter baumannii*

Acinetobacter baumannii (*A. baumannii*), a Gram-negative bacterium, poses very serious health risk as an opportunistic pathogen and is known for the role it plays in multidrug-resistant hospital-acquired infections. It is a non-fastidious, non-fermentative, and non-motile coccobacillus that is a catalase-positive and oxidase-negative bacterium recognized as an ESKAPE pathogen. Its class is Proteobacteria within the Moraxellaceae family and the *Acinetobacter* genus. Its ability to adhere to both

biotic and abiotic surfaces has made it a major focus in biofilm-associated infections which are often resistant to antimicrobial agents.[75]

xix. *Enterococcus faecium*

Enterococci are commonly linked to biofilm-associated infections owing to their widespread presence in the human gut microbiota and their ability to bind to living and non-living surfaces. They are opportunistic pathogens belonging to the group of ESKAPE pathogens. Their attachment to living and non-living surfaces results in catheter-associated urinary tract infections, wound infections, and infective endocarditis —all of which are associated with biofilm formation. Due to their inherent tolerance to antimicrobial agents, enterococcal biofilms present a significant challenge in the treatment of infections.[76]

x. *Klebsiella pneumoniae*

Klebsiella pneumoniae, a capsule forming Gram-negative bacterium, is known to cause a wide range of infections including hospital and community-acquired infections. It has the ability to form biofilms by colonizing living and non-living surfaces such as the mucosal surfaces and medical instruments. Individuals at higher risk for *Klebsiella pneumoniae* infections include newborns, the elderly, and immunocompromised persons. Over the years, the rapid emergence and distribution of multidrug-resistant *Klebsiella pneumoniae* strains has posed a significant global health risk, leading to high rates of morbidity and mortality. *Klebsiella pneumoniae* biofilms have acquired significant resistance strains that have made their eradication difficult. *Klebsiella pneumoniae* is one of the ESKAPE pathogens, and its infections have proved resistant to antimicrobial agents.[77]

xi. *Helicobacter pylori*

H. pylori, a Gram-negative bacterium, is one of the global health threat pathogens responsible for bacterial infection of the gastrointestinal tract. It colonizes the stomach lining and can persist as a chronic infection if left untreated. *H. pylori*'s ability to colonize the stomach lining increases its ability to form biofilms, with infection rates exceeding 80% in developing countries and falling below 40% in developed nations. The bacterium is implicated in various conditions, including chronic gastritis, peptic and duodenal ulcers, and gastric cancers, and has been found to be resistant to antibiotics.[78]

3.2 Fungal biofilms: clinical relevance and resistance mechanisms

i. *Candida albicans*

Candida albicans, a dimorphic fungus, is a common harmless component of the human microflora. It is the most prevalent fungal species in the human microbiota, residing in the gastrointestinal and genitourinary tracts without causing disease.[79–81] However, it can become invasive and pathogenic when it shifts to its hyphal form, which are all enveloped by an extracellular polymeric substance (EPS).[82,83] The biofilms formed by this species are complex assemblies of hyphal cells found on inanimate surfaces and animal tissues. *C. albicans* is often linked to infections on medical devices and it colonizes various host tissues.[84]

ii. *Candida auris*

Candida auris is an emerging fungal pathogen of significant clinical concern due to its multidrug resistance, propensity for causing severe infections, and ability to cause nosocomial outbreaks in healthcare settings. Since its initial identification in 2009, this organism has rapidly disseminated globally, presenting a substantial challenge to public health.[85] *Candida auris* is a pathogenic yeast primarily affecting immunosuppressed patients and individuals with implanted medical devices. *Candida auris* is frequently transmitted from person to person or through contaminated environments, equipment, and tools.[86] They cause hospital outbreaks of candidemia and/or invasive candidiasis in patients admitted to intensive care units.[87–89]

iii. *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*

Non-albicans *Candida* species, *Candida glabrata*, *Candida tropicalis*, and *Candida parapsilosis* are of significant clinical importance due to their ability to form biofilms and their potential resistance to antifungal treatments.[90] Non-albicans *Candida* species, including *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*, are increasingly recognized as significant human pathogens. While less prevalent than *C. albicans*, these organisms pose a growing threat to immunocompromised individuals due to their rising incidence and developing resistance to antifungal treatments.[91]

iv. *Aspergillus fumigatus*

Aspergillus fumigatus is a ubiquitous environmental fungus, commonly residing in soil or organic matter. An opportunistic pathogen, *A. fumigatus* and other filamentous fungi grow as networks of filamentous hyphae that have characteristics of a classic microbial biofilm.[92,93]

A. fumigatus disseminates widely through airborne spores (conidia), with humans inhaling numerous spores daily. It forms biofilms in the lungs, particularly in immunocompromised individuals, and patients with cystic fibrosis or chronic obstructive pulmonary disease (COPD), causing pulmonary aspergillosis.[94,95] *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus* are also among the most common pathogenic species.

v. *Cryptococcus neoformans*

Cryptococcus neoformans is an encapsulated yeast-like fungus found worldwide, and forms biofilms on medical devices and surfaces causing infection in immunocompromised individuals such as HIV patients, organ transplant patients, patients with hematological malignancies, diabetes patients, *etc*.[96,97] The pathogenesis usually originates from *Cryptococcus neoformans* through inhaling spores and small infective particles, ultimately resulting in respiratory infection.[98]

vi. *Malassezia* spp.

Malassezia are small thick-walled ovoid, ellipsoid or cylindrical commensal yeasts of warm-blooded vertebrates, inhabiting the skin of various domestic and wild animals, and are

capable of producing a biofilm that plays an important role in antifungal resistance.[99,100] *Malassezia* spp., formerly known as *Pityrosporum*, are important skin commensals and opportunistic skin pathogens associated with skin infections, and are capable of causing dermatitis (such as atopic dermatitis, folliculitis, and psoriasis) and conditions without inflammation (Pityriasis versicolor), and otitis in humans and animals.[101,102]

vii. *Histoplasma capsulatum*

Histoplasma capsulatum is a dimorphic fungus that causes a fungal respiratory disease called Histoplasmosis. The pattern of its infection in epithelial cells was characterized as a compact mass of yeast cells, which possibly leads to the formation of a complex three-dimensional architecture of biofilms, a phenotype that can induce resistance and enhance virulence, and which can adhere to host tissues and promote the internalization of yeast into host cells.[103–105]

viii. *Scedosporium boydii*

Formerly known as *Pseudallescheria boydii*, *Scedosporium boydii* is an opportunistic fungal pathogen isolated from soil near polluted and clean parts of streams and ponds. It forms biofilms capable of causing a wide spectrum of human diseases, resulting in increased resistance to azole antifungal drugs, making management and treatment of fungal diseases like osteomyelitis difficult.[106,107]

Other ubiquitous opportunistic fungi pathogens causing human diseases and infections due to biofilm formation and antifungal drug resistance include *Trichosporon* spp., *Fusarium* spp., *Coccidioides immitis* and *Coccidioides posadasii*.[108–110]

3.3 Role played by biofilms in disease pathogenesis and infection

Bacterial biofilms are thought to be involved in roughly 65% of all bacterial illnesses.[111] In bacterial infections, the existence of biofilms increases the pathogenicity of the bacteria and shields them from being eliminated by outside therapy.[112] Since bacteria that live in biofilms can be resistant to the immune system, antibiotics, and other treatments, biofilm infections are usually chronic.[113]

The matrix of exopolysaccharides (EPS) is essential to the composition of biofilms and contributes to their pathogenicity. For example, *P. aeruginosa* biofilms contain alginate, a polyanionic exopolysaccharide made of uronic acid. However, polysaccharide intercellular adhesin (PIA), a polycationic exopolysaccharide, is what makes up biofilms in *S. aureus* infections.[114] The lipopolysaccharide (LPS) layer of *P. aeruginosa* biofilms is a crucial virulence component that cells recognize through the Toll-like receptor TLR4. The EPS layer of the bacterium efficiently covers this layer and conceals it from immune identification.[115] *P. aeruginosa* changes into a mucoid phenotype during infections in cystic fibrosis (CF) patients, which is characterized by an excess of alginate. This phenotype boosts *P. aeruginosa*'s resistance to antimicrobial therapies, predators, and host defenses.

The infections caused by biofilms are varied. These include endocarditis, infections in cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses, heart valves, and intervertebral discs. They also include less common but more deadly conditions like bacterial vaginosis, urinary tract infections, catheter infections, middle ear infections, dental plaque development, gingivitis, and coating contact lenses. Many devices have had data on device-related infections assessed, with results including rates of 2% for joint prosthesis; 2% for breast implants; 4% for mechanical heart valves; 10% for ventricular shunts; 4% for pacemakers and defibrillators; and roughly 40% for ventricular-assisted devices.[116]

To effectively colonize an area and produce illnesses and infections, biofilms must go through specific stages of regulation.

Biofilms have been linked to several potential functions in the pathophysiology of disease. Vestby *et al.*[113] described such roles: (i) biofilms enable intracellular invasion, cause local inflammation, and impede healing by erecting a physical barrier; (ii) chronic subclinical infections may result from bacteria's long-term protection in biofilms, and other pathogens may use pre-existing biofilms to evade immune system destruction; (iii) biofilms enhance bacterial resilience to antimicrobial treatments and the body's defense mechanisms; (iv) biofilms may serve as an ecosystem where bacterial species and populations are concentrated in specific areas; (v) this can have detrimental effects on host cells due to concentrated, sequential, and/or synergistic activities of present bacteria; (vi) persistent biofilms may modify the local immune response, inducing or exacerbating tissue damage through local inflammation.

3.4 Mechanisms of antimicrobial resistance in biofilm niches

Upon frequent exposure to antibiotics, bacteria can become resistant to the antimicrobials genetically.[117] One of the other ways in which bacteria evade antibiotic exposure is *via* the formation of biofilms.[118]

i. Biofilms' extracellular matrix acts as a barrier, limiting the effectiveness of antibiotics

This extracellular matrix acts as a chemical and physical diffusion barrier to most antibiotics, retarding their penetration into the biofilm and to the target site, thus making them less effective.[119,120] Components of the Extracellular Polymeric Substances (EPS) include polysaccharides, proteins, extracellular DNA, lipids, humic substances and metabolites. Polysaccharides play a crucial role in maintaining biofilm integrity as the primary EPS component. These exopolysaccharides are essential structural elements in microbial biofilms and include poly-*N*-acetylglucosamine (dPNAG), alginate, Psl, Pel, amylose-like glucan, cellulose, galactosaminogalactan, β-(1,3)-glucan, levan, and inulin.[121] Glycocalyx, consisting of glycoproteins and polysaccharides, favours biofilm attachment to surfaces, helps in biofilm maturation, and aids microorganism survival in unfavourable host environments. It can accumulate antibacterial agents up to 25% of its weight, limit microbicide transport, and attach exogenous substances that degrade antibiotics.[122] Different bacteria produce distinct extracellular matrix (ECM) components. *Pseudomonas aerugi-*

nosa is known for its polysaccharide alginate-, Pel- and Psl-rich ECM.[123] Alginate protects *Pseudomonas* biofilms from aminoglycosides, while Pel protects against aminoglycosides, tobramycin and gentamicin.[124] Psl binds eDNA and contributes to resistance against colistin, polymyxin B, tobramycin, and ciprofloxacin in early biofilm formation stages.[125,126] Antibiotic binding to biofilm components results in decreased antibacterial activity, mostly through antibiotic depletion.[119]

ii. Altered microenvironment and slow growth rate

Biofilms create gradients of nutrients, oxygen, and waste products, leading to a heterogeneous microenvironment. This results in some bacteria within the biofilm entering a dormant or slow-growing state, which makes them less susceptible to antibiotics that target actively dividing cells.[127]

iii. Enzyme-mediated resistance

In a biofilm, some of the microorganisms that make up the community produce modifying enzymes that reside within or near the cell surface, which selectively target and inactivate antimicrobials.[128] The transformation or modification of antimicrobials to the nontoxic form *via* hydrolysis and ion redox reactions are mediated by enzymes that provide resistance to biofilm; this is known as enzymatic inactivation. It is achieved through complete drug molecule destruction or the addition of chemical groups to the drug. β-Lactamases are the most common example of drug-destruction enzymes. They destroy the drug by hydrolyzing a site in the ring structure of β-lactam drugs.[129] In the case of the transfer of chemical groups, the most diverse and largest family of resistance enzymes is the group transferases. These enzymes covalently modify antibiotics leading to structural alterations that impair target binding. Chemical strategies include *O*-acylation and *N*-acylation, *O*-phosphorylation, *O*-nucleotidylation, *O*-ribosylation, *O*-glycosylation and thiol transfer. Acetyl, adenyl, and phosphoryl groups are some of the most transferred chemical groups and acetylation is the most common reaction.[122,130,131]

iv. Quorum sensing in biofilm interaction

Quorum sensing (QS) enables bacteria to communicate and coordinate their behaviour, including the development of resistance mechanisms.[132] It is a process of cell-to-cell interaction that regulates the behaviour of bacteria. It depends upon extracellular signal molecules, detection, production, and autoinducers.[133] The function of these systems depends solely on the bacterial density (density-dependent) and through them the bacteria regulate the expression of various genes and the production of infectious agents such as extracellular enzymes and lysines.[134] These are necessary for the pathogenicity of infections, but also affect antibiotic resistance, inflammatory response and biofilm development.[122] Three types of bacterial QS systems are known with particular self-inducing molecules, which include the QS system with acyl-homoserine lactone (AHL) found in Gram-negative bacteria. Oligopeptides (AIP) are found in Gram-positive bacteria. The third type is the QS system with furan borate diesters and is found both in Gram-negative and Gram-positive bacteria.[121,135]

v. Genetic adaptation of biofilms to maintain resistance

Microorganisms must be capable of both vertical inheritance and horizontal gene transfer (HGT) of resistance genes to maintain antibiotic resistance.[136] The proximity of cells within a biofilm facilitates horizontal gene transfer through the provision of compatible conditions such as high cell density, increased genetic competence and accumulation of resistance genes; transfer includes the exchange of plasmids carrying antibiotic resistance genes.[14,137,138] Biofilms facilitate the close proximity of bacterial cells, enhancing the likelihood of HGT through mechanisms such as transformation, transduction, and conjugation. Bacteria within biofilms can acquire plasmids carrying antibiotic-resistance genes through conjugation.[136]

vi. Role played by persister cells in biofilm-mediated antibiotic resistance

Biofilms harbour a subpopulation of persister cells (classically <1%);[139] they are dormant variants, though genetically identical to the active cells and can survive high concentrations of antibiotics.[72] These cells are not resistant in the genetic sense but can regrow once the antibiotic treatment is stopped, leading to recurrent infections. It is now thought that the main contributor to the increased antimicrobial resistance of biofilms is a subset of cells known as persisters, a small fraction of the bacteria populations that survive lethal doses of antimicrobials without any resistance mechanism.[140]

vii. Efflux pump-mediated tolerance in biofilms

Efflux pumps are membrane proteins which expel substances toxic to microbial cells from the inside of the microbial cell to the outside environment.[141] Some bacteria within biofilms upregulate efflux pumps, which actively expel antimicrobial agents from the cell. Efflux pumps aid in the development of antimicrobial resistance within biofilm communities. There are six superfamilies of the efflux pump;[142,143] they are:

i. Major Facilitator Superfamily (MFS).

ii. Resistance-Nodulation-Cell Division (RND) superfamily.

iii. ATP-Binding Cassette (ABC) transporters superfamily.

iv. Small Multidrug Resistance (SMR) superfamily.

v. Multidrug and Toxic Compound Extrusion (MATE) superfamily.

vi. Proteobacterial Antimicrobial Compound Efflux (PACE) superfamily.

In species of Gram-negative bacteria like *Pseudomonas aeruginosa* and *Escherichia coli*, the Resistance-Nodulation-Cell Division (RND) superfamily is of particular interest in biofilm-mediated resistance; the MexAB-OprM and AcrAB-TolC efflux systems are well-characterized efflux pumps that belong to the Resistance-Nodulation-Cell Division superfamily.[144] The MexAB-OprM efflux system is a well-studied RND-type efflux pump in *Pseudomonas aeruginosa*. It can expel various antibiotics, such as beta-lactams, fluoroquinolones, tetracyclines, and chloramphenicol, contributing to multi-drug resistance.[145] The AcrAB-TolC efflux system is another RND efflux pump found in *Escherichia coli*. It can expel various antibiotics, such as quinolones, beta-lactams, chloramphenicol, tetra-

cyclines, and some dyes and detergents, thereby contributing to multi-drug resistance.[146]

The efflux pump system is a non-specific transport mechanism that is capable of expelling a wide range of antibiotics therefore allowing biofilm-forming bacteria to exhibit multi-drug resistance (MDR), making treatment of infections with standard antibiotic therapies very challenging.[147]

3.5 Clinical implications of biofilm colonization, challenges in treatment and control of infection

Because they allow bacteria in consortia to avoid antibiotic treatment, leading to treatment failures and selection of resistant strains, biofilms are clinically relevant. Biofilm colonization has been reported on both living and inanimate surfaces.

Catheter-related bloodstream infections (CRBSIs) are primarily caused by dead ends, which encourage biofilm growth. Catheter biofilm formation has been linked to the most frequent nosocomial infections with serious side effects. They may cause persistent bloodstream infections, septicemia, and endocarditis, or act as a reservoir for transferring germs to other body parts.[42] Biofilms have been linked to approximately 65% of nosocomial infections, including serious hospital-acquired infections from indwelling catheters and prosthetics.[148]

A recent study found that polymicrobial biofilm including multidrug-resistant organisms (MDROs) could persist on furnishings and equipment in an intensive care unit for up to a year, even after terminal cleaning.[149] The ESKAPE pathogens are a significant contributor to biofilm-mediated infections, accounting for over 40% of infections in critical care patients.[149] Antibiotic resistance is a significant global clinical issue when treating nosocomial and community-acquired illnesses caused by ESKAPE bacteria.[150] A study on 8756 clinical samples in a Nepalese hospital showed a high prevalence of antimicrobial resistance and biofilm formation in ESKAPE isolates.[151] Every member of the ESKAPE pathogen group is on the WHO's important and high-priority list of pathogens for antimicrobial research.[152] Studies show 60–90% of chronic wounds have biofilms containing various pathogenic bacteria, including fungi and ESKAPE group bacteria.[42]

Several *in vitro* biofilm models have demonstrated the clinical relevance of biofilms. However, most do not convert to *in vivo* models, impacting treatment of biofilm-mediated illnesses. Skin infections, oropharyngeal infections, cystic fibrosis, and infections related to implants and devices have been used to explain the clinical relevance of *in vitro* biofilm models.[152] Nevertheless, *in vivo* model data are still lacking. Several effects of biofilms on human health, including reduced antibiotic treatment efficacy, host immune response evasion, delayed wound healing, dental disease, cancer development promotion, initiation or aggravation of autoimmune disease, and colonization resistance, have been reviewed and are supported by experimental data or clinical observations.[153] According to the Kyoto Global Consensus report, *Helicobacter pylori* gastritis was for the first time fully classified as an infectious disease.[154] The stomach mucosa's *H. pylori* biofilm organization has been observed.[155] The report notes that the creation of biofilms that result in treatment failures has been one of the problems impeding the success in eliminating *H. pylori*, among other reasons. There is evidence of a relationship between *H. pylori* biofilm and antibiotic resistance.[156,157] Unless there are strong medical justifications, the Kyoto Global Consensus report on *H. pylori* gastritis suggests eliminating all *H. pylori* infections. However, in recent years, efforts to eradicate *H. pylori* have faced an increasing challenge due to the increased rate of antibiotic resistance.

4 Advances in biofilm control strategies

4.1 Natural products in biofilm control, therapeutic potential and mechanistic insights

An estimated 90% of bacteria are present as biofilms and significantly impact bacterial infection. The increase in multi-drug resistance incidence over time has led to the development of novel interventions other than conventional therapy to manage the virulence activities of biofilms as well as prevent their pathogenesis.[158] The tolerance of antibiotics to biofilms has posed a significant global challenge across various health sectors. This is attributed to the inability of infectious diseases linked to biofilms to be treated by synthetic drugs and conventional and combined antibiotics. Biofilm-linked infections are difficult to treat due to the presence of multi-drug-resistant microbes. These agents cannot achieve the eradication of biofilms, hence; the search for novel natural antimicrobial/anti-biofilm agents.[159] The *in vivo* toxicity of known antibiotics, low efficacy and the ability to readily develop resistance have been the drivers for researchers to discover effective, low-concentration, cost-effective natural agents for biofilm eradication.[21] Natural products with lesser side effects are perceived as more efficient anti-biofilm agents compared with synthesized/chemical compounds. These natural products are perceived to be non-toxic and appear harmless for both the human body and the environment; hence, their use as potential anti-biofilm agents for application in various fields of research. These natural approaches include phytocompounds, antimicrobial peptides, bacteriophages, biosurfactants, antimicrobial photodynamic therapy (aPDT), gene editing by CRISPR-CAS, and nano-mediated techniques. Natural anti-biofilm agents act either solely or synergistically by diverse modes of action.[160]

4.1.1 Plant-based agents (phytocompounds). Plants are known to represent a vast, sustainable resource of diverse classes of low molecular weight compounds with various pharmacological activities. Novel natural approaches have explored phytochemicals extracted from plants to prevent biofilm formation. These reported activities of plant-based anti-biofilm agents are evaluated based on any one of the mechanisms leading to biofilm formation, and broadly comprise essential oils, phenolic compounds, terpenoids, lectins, alkaloids, polypeptides, and polyacetylenes.[161] Phytocompounds

have been used either in combination or alone to repurpose resistant antibiotics for anti-biofilm activity. Plant extracts have likewise been shown to control biofilm advancement and hinder quorum sensing (QS) in bacteria.[162] Different subclasses of phenolic compounds exist including phenolic acids, quinones, flavonoids, flavones, flavonols, coumarins and tannins. Coumarins, as naturally derived fused benzene and a-pyrone rings, are prevalently present in plants with antibiofilm activity. Warfarin, ellagic acid, nodakenetin, Girennavar and fraxin are notable compounds obtained from coumarin that have shown anti-biofilm activity. Furan molecules known as furocoumarins extracted from grapefruit showed comparable activity to coumarins and inhibited biofilm development in Gram-negative organisms. It has been discovered that tannins, especially condensed tannins, have anti-biofilm activity.[163–165]

Plant-based compounds act as anti-biofilm agents by mechanisms which include quorum sensing inhibition, substrate deprivation, membrane disruption, binding to adhesin complex and cell wall, binding to proteins, interacting with eukaryotic DNA, and blocking viral fusion.[161,166]

Numerous medicinal plants have been exploited that have promising anti-biofilm activity when extracted using a wide range of solvents. These include *Fritillaria verticillata*, *Liriope platyphylla*, *Cocculus trilobus*, *Cinnamomum glaucescens* (Nees) Hand-Mazz, *Rhus verniciflua*, *Ginkgo biloba*, *Syzygium praecox* Roxb. Rathakr. & N. C. Nair, *Bischofia javanica* Blume, *Elaeocarpus serratus* L., *Smilax zeylanica* L., *Trema orientalis* (L.) Blume, *Acacia pennata* (L.) Willd., *Holigarna caustica* (Dennst.) Oken, *Murraya paniculata* (L.) Jack, *Pterygota alata* (Roxb.) R. Br, and Curcumin from *Curcuma longa* (turmeric). A Chinese herb, Herba patriniae, has prevented the gene expression of six genes linked with biofilm development and EPS production in *P. aeruginosa.*[158,163,165] Ginkgolic acid, an extract from *Ginkgo biloba*, has shown various medicinal properties such as antitumor, antimicrobial, and neuroprotective activity, and is also active against *S. aureus* strains and *E. coli* biofilm formation. Similarly, plants like *Aloe vera*, *Melaleuca alternifolia*, *Aspalathus linearis*, *Camellia sinensis*, *Glycyrrhiza glabra*, *Hypericum perforatum*, *Leptospermum petersonii*, *Agathosma betulina*, and *Syzygium aromaticum* have all been reported to exhibit anti-biofilm activity.[167]

Extracts from these plants have promising anti-biofilm activity against wide range of bacteria: *S. aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Serratia marcescens*, and *Proteus mirabilis etc*. *via* slowing the bacterial motility.[158] Harjai *et al*. reported anti-biofilm agents from natural compounds, such as 1,2-benzene- dicarboxylic acid, diisooctyl ester which assumes a critical role in hindering bacterial suppression and stifling biofilm-related genes.[165] *Salvia officinalis* L., an Algerian plant, and garlic (*Allium subhirsutum* L.) extracts have been reported to have *in vitro* anti-biofilm activity against different bacterial and fungal species.[75] The genes involved during the formation of biofilms are suppressed and their adhesion inhibited by interfering forces of compounds present in phytocompounds. The bacterial density present in the biofilm matrix was significantly reduced when *Eruca sativa* Miller was tested against various food-borne pathogens for its antibacterial and antibiofilm action. *Eruca sativa* was shown to exert exopolysaccharide synthesis inhibition. Phytochemicals have the potential to interfere with the extension along with the capability to stop the accessibility to nutrients essential for adhesion and bacterial growth.[158,168]

Reports have shown the ability of clove bud oil to inhibit biofilm formation and disrupt the preformed biofilms of certain bacteria. Kalia *et al.* prepared a concentration of 1% clove bud oil which reduced biofilm formation by 85.3% while promoting biofilm dispersal by 50.4%.[169] Ajoene, a sulphur-containing extract from *Alluim sativa*, exhibited antibiofilm activity against varieties of Gram-negative bacteria such as *Klebsiella pneumoniae*, *Escherichia coli*, and *Xanthomonas maltophilia.*[119] Eugenol has been active as a phytocompound against virulence factors of *Chromobacterium violaceum* and *Pseudomonas aeruginosa* strains by inhibiting quorum sensing-mediated violacein. Its anti-biofilm effectiveness has also been reported by Ta *et al*. against clinical isolates of *Listeria monocytogenes* and *Klebsiella pneumonia.*[170]

Quercetin, a flavonoid from plants, was also found to inhibit a stage in biofilm formation in certain foodborne pathogens such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Yersinia enterocolitica.*[171] Kumar *et al*. reported compounds including zingerone isolated from *Zingiber officinale* (ginger) that inhibited quorum sensing of signal molecules in clinical isolates of *Pseudomonas aeruginosa.*[170,172] Asma *et al*. reported the anti-biofilm activity of *Chamaemelum nobile*, a naturally occurring well-known plant against *P. aeruginosa* biofilm by disrupting its self produced matrix. Proanthocyanidins extracted from cranberries have significantly inhibited the adhesion of *E. coli* to uroepithelial cells and biofilms formed by *Streptococci* spp.[173] The extracts of *Acalypha wilkesiana*, *Encephalartos laurentianus*, *Cinnamomum burmanii*, *Artemisia arborescens*, *Paederia foetida*, *Sophora secundiflora*, *Sphaeralcea ambigua*, *Prosopis laevigata*, *Opuntia ficus-indica*, *Dioon spinulosum*, *Marrubium vulgare*, *Cymbopogon* spp.(*Cymbopogon proximus* and *Cymbopogon citratus*), *Scutellaria drummondii*, *Nothoscordum bivalve*, *Apium graveolens*, *Plantago ovata*, *Vitis vinifera*, *Viscum album*, *Senna acutifolia*, *Melissa officinalis*, and *Gutierrezia microcephala* exhibited anti-biofilm activity against multiple species of microorganisms.[173,174] Similarly, Teanpaisan *et al.* reported the anti-biofilm activity of *Piper betle* against mutans of *Staphylococcus*, ATCC 25175 and *A. actinomycetemcomitans* ATCC 33384.[175] *Rosmarinus officinalis*, *Echinacea angustifolia*, *Thymus vulgaris* and *Mentha piperita* extracts exhibited the ability to reduce attachment of *Listeria monocytogenes* ATCC 19111 biofilms by at least 50%.[168]

Phytochemicals from citrus plants have been reported by Vikram *et al*. to regulate biofilm formation and inhibit virulence factors of *E. coli* O157:H7, with a flavonoid (naringenin) as the potent nonspecific inhibitor of cell–cell signaling. Flavonoids, particularly naringenin, quercetin, sinensetin, and apigenin from citrus, are inhibitors of cell–cell signaling and biofilm formation. Also, limonoids from citrus exhibited the

Table 1 Some plant-based anti-biofilm agents

Plant source	Active compound/extract/ essential oil	Pathogenic specie	Ref.
Musa acuminate	5-Hydroxymethylfurfural	*P. aeruginosa*	177
Syringa oblate	Syringopicroside	*Streptococcus suis*	178
Humulus lupulus	Xanthohumol	*S. aureus*	179
Cymbopogon citratus	Lemon grass Essential oil, Citral	*P. aeruginosa* and *Staphylococcus aureus*	180
Allium sativum (garlic)	Ajoene and Allicin	*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *E. coli*, *L. monocytogenes*, and *Acinetobacter baumannii*	181 and 182
Syzygium aromaticum (clove)	Eugenol	*Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Klebsiella pneumoniae*	170 and 183
Allium cepa (onions), *Malus domestica* (apple)	Quercetin	*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Yersinia enterocolitica*	171
Quercus cortex (Oak)	Bark extract	*Chromobacterium violaceum*	184
Cinnamomum zeylanicum	Essential oil	*E. coli* and *S. epidermidis*	185
Thymus vulgaris	Essential oil	*Acinetobacter baumanii*, *Citrobacter freundii*, *Corynebacterium striatum* and *E. coli*	186
Eugenia caryophyllata	Essential oil	*Klebsiella* spp., *S. aureus*, *Salmonella* spp. and *P. aeruginosa*	187
Azadirachta indica	Ethanol extract	*MRSA*	188
Moringa oleifera		*MRSA*	
Psidium guajava	Ethanol extract and petroleum extract	*MRSA*	188
Acacia nilotica	Aqueous extract	*E. coli*, *P. aeruginosa*, and *K. pneumoniae*	189
Citrus spp (*Citrus grandis*, *Citrus hystrix* and *Citrus reticulate*)	Essential oil	*E. coli* and *S. epidermidis*	185
Vaccinium macrocarpon Aiton (Cranberry)	Polyphenols	*E. coli*, *P. aeruginosa*, and *K. pneumoniae*	190
Tessaria absinthioides	Extract	*Bacillus* spp. and *Staphylococcus* sp.Mcr1	191
Curcuma longa L.	*Curcumin*	*Acinetobacter baumannii* and *C. albicans*	192
Rheum officinale Baill	Aloe-emodin	*Staphylococcus aureus*	193
Vaccinium macrocarpon Aiton (Cranberry)	Vanilic acid, protocaterchuic, catechin	*E. coli*	190
Hordeum vulgare L. (sprouting)	Hordenine	*Pseudomonas aeruginosa*	194
Zingiber officinale Roscoe	Zingerone	*Pseudomonas aeruginosa PAO1*	195

ability to inhibit *V. harveyi* biofilms. Table 1 reveals certain plant-based anti-biofilm agents.[176]

Several researchers have studied Bee products as agents with a wide range of antibacterial, antioxidant, antiviral, antifungal, anticancer activities *etc*. The use of honey and its bioactive components against wide range of planktonic and sessile bacteria has been reported.[196]

The ethanolic extract of propolis from certain regions of Hungary in combination with vancomycin, evaluated for antibiofilm activity by Bouchelaghem *et al.*, exhibited its effectiveness against MSSA and MRSA. This combination significantly led to the degradation and disruption of mature biofilms.[197] The antibiofilm activity of various honey samples (Manuka honey (from New Zealand), Buckwheat and Canadian clover honey (from Canada), and Sidr honey (from Yemen)) were also evaluate by Alandejani *et al.* The honey samples demonstrated activity against biofilms formed by bacteria such as *P. aeruginosa*, methicillin-resistant *S. aureus* (MRSA), and methicillin-sensitive *S. aureus* (MSSA). Similarly, Manuka honey exhibited antibiofilm activity against extended spectrum β-lactamase (ESBL) and carbapenemase-producing *K. pneumoniae*, ESBL-producing *E. coli*, multidrug-resistant (MDR) *P. aeruginosa*, resistant strains of *Ureaplasma urealyticum*, and *Ureaplasma parvum*.[198]

4.1.2 The emergence of enzyme-based and peptide-based therapies: antimicrobial peptides (AMP). Recently, researchers have highlighted anti-microbial peptides among various anti-biofilm strategies. AMPs, also known as host defense peptides, are increasingly recognized as promising approaches for biofilm eradication, due to lower susceptibility to causing resistance compared with conventional /traditional antibiotics.[196] Antimicrobial peptides are natural anti-biofilm agents derived from microorganisms, animals and plants, with a varying number of amino acids.[199] AMPs are oligopeptides recently reported to reduce and inhibit bacterial biofilm formation by disruption of the cell membrane, which disturbs the transmembrane pore mechanism and finally results in cell death. The cationic charge and the hydrophobic moiety of peptides are responsible for their interaction with the cell membrane. Strangely, certain AMPs attach to the bacterial cells of the biofilm structure, empowering its agglutination and membrane communication. Various factors are highlighted as key mechanisms, such as amphipathicity, amino acid composition, size affecting peptide attachment and translocation, and altering of membrane permeability through an alteration in cytoplasmic membrane configuration.

AMPs occur naturally in plants, animals, microorganisms, and humans and act by electrostatically interfering with membrane phospholipids on bacterial cell membranes, followed by insertion into the membrane, thus killing bacteria. Doiron *et al*. derived five natural antimicrobial peptides from marine snow crabs which were effective against *Pseudomonas aeruginosa* biofilms.[199,200] The antimicrobial peptides polymyxin B

and colistin have been observed to reduce the resistance of *P. aeruginosa* biofilm infections. An example of an antimicrobial peptide derived from microorganisms that exhibited the potential to eradicate antibiotic-resistant biofilms is bacteriocin. Bacteriocin has been employed either alone or in combination, and its component, nisin A, has been improved using bioconjugation with silver nanoparticles. This improved version has been effective in disrupting the membrane of methicillin-resistant *Staphylococcus aureus* (MRSA) strain biofilm-embedded cells.[201] A human peptide Cathelicidin, which can inhibit *Pseudomonas aeruginosa* biofilm formation through downregulating quorum sensing and decreasing the attachment of bacterial cells on the surface, has also been reported.[172]

The potential of antimicrobial peptides to disrupt biofilms that have developed on medical devices (catheters, artificial valves, stents, dentures) has been reported. Hospital-acquired infections of ESKAPE and non-ESKAPE pathogens (*S. aureus*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Enterococcus faecium*, *Acinetobacter*, and *Enterobacter* spp.) form biofilms which are disrupted by AMPs.[202] AMPs are less vulnerable to microbial resistance and are preferred to traditional antibiotics. Shahrour detailed the synergism between AMPs and other antimicrobial agents in suppressing several resistance pathways of biofilm formation.[203] The animal source of AMP known as amphibian skin is effective against various biofilm-causing microorganisms. An AMP Japonicin-2LF, which acts as a detergent and was isolated from Fujian large-headed frog (*Limnonectes fujianensis*) skin secretion, exhibited inhibition activity against MRSA biofilms through membrane permeabilization. Japonicin-2LF's anti-biofilm activity has been reported against both planktonic and sessile pathogens. Similarly, esculentin-1a, *i.e.*, Esc (121) and its D-amino acid-containing diastereomer Esc(1-21)-1c from frog skin inhibited *P. aeruginosa* biofilm formation by decreasing its swimming, swarming, and twitching motility. The peptide melittin, isolated from bee venom, exhibits antibacterial activity and is utilized in the prevention of MRSA systemic infections. Human Beta-Defensin 2 peptide has also been reported to possess inhibitory activity against *P. aeruginosa* biofilm by inducing structural changes, altering the outer membrane protein profile and interfering with the transfer of biofilm precursors into the extracellular space. AMPs, as proteins/peptides, are prone to degradation by bacterial proteases, hence their effectiveness in treating biofilm-based infections is limited.[204]

Reports have described more than 3000 AMPs but approval by the FDA has only been obtained for about seven of them. This signifies a serious scarcity of clinical studies on natural AMPs owing to factors such as unpredicted side effects, poor performance, cytotoxic and hemolytic activities, *etc*. The failure of natural AMPs in pre-clinical stages might be because of the differences between the clinical setting and their natural resident conditions. Thus, there is a need for improvement in clinical research for these natural anti-biofilm agents against different antibiotics tolerant to biofilms. It is vital to exploit the design and structure of different natural AMPs to foster novel therapeutic peptides with further developed stability and activity in correlation with their natural partners (Fig. 5).[166]

4.1.3 Bacteriophages: growing interest against antibiotic resistance. Bacteriophages are regarded as promising tools for combating the growing problems and crisis of antibiotic resistance on a global scale. Bacteriophages, commonly known as phages, are bacterial viruses that infect, suppress, and kill

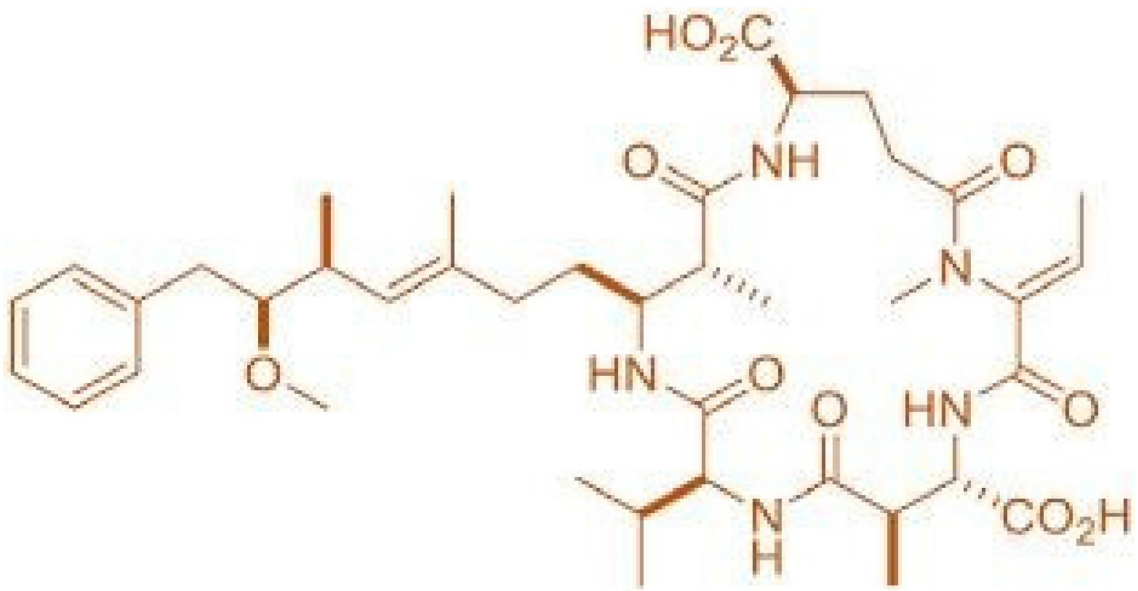

Fig. 5 Structure of antimicrobial peptides. Reproduced from ref. 174 with permission from BioMed Central, Springer Nature, London, United Kingdom, copyright 2017.

other types of bacteria while leaving specialized bacterial and human cells unharmed.[205] As the most prevalent biological entities in the biosphere, phages have been used as a possible treatment for multi-drug resistant (MDR) diseases since the 1990s. They have been used as an effective therapeutic strategy to remove biofilm cells. In 2020, a study by Gharieb *et al.* involved two lytic phages, namely vB_SauM_ME18 and vB_SauM_ME126, that had promising anti-biofilm potential against multi-drug resistant *S. aureus* biofilms.[206] Again, enzymes produced by phages have also been employed as therapeutic options to eradicate biofilms by destroying the exopolymeric matrix of biofilms.[207] This was achieved by the penetration of the biofilm layers through the pores and channels. Phage-encoded lytic enzymes, such as depolymerase, holins, and endolysins, can break down bacterial polysaccharides and rapidly break down biofilms, enabling phage invasion of the cells inside the inner biofilm layers.[208,209] There have been classes of depolymerase enzymes (hydrolases and lyases) identified in about 143 phages. These enzymes identify, attach to, and break down the EPS matrices of biofilms, causing structural disruption. Peptidoglycan hydrolases known as endolysins are produced at the end of an infection cycle and cleave to the peptidoglycans found in the cell wall.[210]

A T7 phage engineered by Pei *et al.* disrupted polymicrobial biofilms by cleaving to the biofilm matrix signalling molecules in an approach known as quorum-quenching. Subsequently, it helps in treating multiple host biofilm infections.[211] Reuter and Kruger found that (engineered) phage-derived enzymes—polysaccharide depolymerase or peptidoglycan-degrading enzymes—are promising therapeutic anti-biofilm candidates.[211,212]

Olsen *et al.* reported the synergistic effect of endolysins and depolymerase DA7 in disrupting the cell membrane of *Staphylococcus aureus* biofilms, thereby leading to their eradication. Biofilms formed on human cystic fibrosis bronchial epithelial cell lines are disrupted by lytic bacteriophages and phage cocktails such as OMKO1, myovirus (ϕNH-4), and podovirus (ϕMR299-2). They are considered powerful approaches for biofilm eradication of *P. aeruginosa* NH57388A (mucoid) and *P. aeruginosa* MR299 (nonmucoid) strains.[93,212,213]

Several phage-based therapeutic options are available and promote phages to maintain human health. It is imperative to develop safe and efficient treatments for antibiotic-resistant biofilm-mediated infections. The School of Medicine, University of California San Diego (UCSD) phage therapy centre hosted the first patients that received phage treatment upon its FDA approval in the year 2019. Despite its approval, it has only been active in a few countries. However, many limitations have been associated with phage therapy including the establishment of phage banks, stability, shortage of phage therapy centres, safety, and quality of phage preparations during production; and the evolution of bacterial resistance to phages.[75,214] Reports have also detected resistance and adverse immune responses to phage therapy as a growing problem which requires further research and thorough investigation.

4.2 Advances and innovations in biofilm control strategies

There have been advances towards the control of microbial biofilms, as various control strategies have recorded appreciable levels of resistance. This has necessitated continuous research into new and effective drugs and drug combinations to fight biofilms. These approaches are increasingly becoming the mainstay in the fight against all biofilms. They include the following.

4.2.1 Antibiotic adjuvants. Broad-spectrum antimicrobial agents approved by the FDA for use in toothpaste and disinfectants are referred to as antibiotic adjuvants. Triclosan, an antibiotic adjuvant, has been shown in studies to work in concert with other traditional antibiotics to eradicate biofilms. Research showed that tobramycin and triclosan increased the effectiveness of tobramycin against clinical isolates of certain *Burkholderia cenocepacia* and *Staphylococcus aureus* biofilms. Similarly, antibiotic adjuvants have been reported to exhibit a synergistic effect with gentamicin and streptomycin against certain strains of bacterial biofilms. The use of antibiotic adjuvants has also found use in the eradication of biofilms formed on medical devices.[215] Consequently, high doses of antibiotic adjuvants employed as topical treatments in combinations of tetracyclines, aminoglycosides, cephalosporins, monobactams, polymyxins, and glycoglycines have been shown to be effective against biofilms. Sharma *et al.* reported that lung infections caused by biofilms are eradicated by combinations of antibiotic adjuvants and inhaled antibiotics which include: colistin, tobramycin, aztreonam, ciprofloxacin, levofloxacin, or gentamicin.[14]

4.2.2 Nanocarriers and smart-drug delivery systems. The science of nanotechnology and nanocarriers towards biofilm eradication emanated from the ability of the biofilm's EPS complex matrix to be penetrated, allowing a complete interaction between matrix and microbial cells involving both susceptible and resistant strains. Nanoparticles (NPs) also interfere with the physical and chemical properties of the biofilm matrix that help to maintain the stable 3D structure of microbial biofilms. Primarily, the small size of nanoparticles allows penetration into the biofilm microenvironment, facilitating disruption of its integrity.[216] The creation of multi-functionalized nanostructured antimicrobial agents with the potential for biofilm elimination has been made possible by developments in the field of nanomedicine. An emerging method for encapsulating bioactive compounds for sustained and site-specific administration is called nanoencapsulation.

A few nanoparticles have been essential in inhibiting biofilm infections of microbes, including silver–silica dioxide nanoparticles ($AgSio_2$ NPs) used as an implant coating, chitosan-capped gold and silver nanoparticles coupled together using the tiger milk mushroom, silica nanoparticles, and nucleic acids nanoparticles. Nanoparticles of smaller sizes (6 nm) have shown more antibiofilm activity than the larger particles (11 nm) owing to their pronounced release of silver ions. However, the larger surface area of nanoparticles influences the increased surface reactivity and increased antibio-

film activity of silica nanoparticles, metal and metal oxide nanoparticles such as copper oxide, ferrite, silver, and titanium dioxide nanoparticles. NPs' hydrophobicity, shape, and surface charge are among other NP-related characteristics that support their antibiofilm activity. When designing NPs that target bacterial biofilms, taking these factors into account can help in the synthesis of those NPs. Nucleic acid nanoparticles can either directly act as an antimicrobial or transfer medicinal agents, like enzymes, essential oils, or phytochemicals, to kill the bacteria within the biofilm. Experimentally and *in vivo*, metal nanoparticles have demonstrated unique control over the bacterial signaling system. By meddling with the QS regulatory genes, metal NPs prevent the synthesis of signaling molecules. In addition, silver NPs integrate themselves within the bacterial DNA, gold NPs trigger ROS-mediated damage, chitosan NPs can invade the EPS matrix and titanium oxide NPs (TiO-NPs) induce EPS lipid peroxidation, and all these cause damage. Again, liposomes obstruct the electron transport systems of bacteria thereby suppressing the QS system.[217]

Recently, a novel strategy brought together nanotechnology and the CRISPR-Cas9 gene-editing method. One study involved the development of a dissolvable patch by Wan *et al*. to treat inflammatory skin conditions. To take advantage of their complementary actions, the researchers employed nanoparticles to transfer glucocorticoids and cas9 (gene editing agents) into the nuclei of cells. Both the *in vivo* findings and the mouse models demonstrated improved glucocorticoid effectiveness and decreased skin inflammation. This work provides more insight into studies that use gene-editing methods to target disease-causing microbes to deliver nanoparticles (NPs) loaded with new antibiotics, existing antibiotics or repurposed antibiotics.[218]

4.2.3 Phage therapy and nanotechnology. The use of bacteriophages in combination with nanotechnology has enhanced their stability, delivery and ability to infect and disrupt biofilms. The use of this combination was first introduced by Esteban *et al*., involving the addition of Bacteriophage-K in an oil-in-water nano-emulsion formula to improve its stability and infectivity. This activity increased its killing activity against *S. aureus* biofilms. Fascinatingly, the phage–nanoemulsion composition increased the phage–bacterial contact and enhanced antibacterial activity by reducing the electrostatic repulsion between the negatively charged bacteria and phage.[219]

Other research by Liu *et al*. reported the conjugation of naturally isolated and purified phages with chitosan film (a biocompatible agent). The nanoparticle combination reduced the significant colonization of medical implants by biofilms and controlled the increase in bacterial infections by phages. The free phages had a reduced effectiveness in the phage–chitosan conjugate compared with the natural phages. Nevertheless, based on SEM imaging, it was calculated that the phages' 79.5% reduction in bacterial density relative to the control was what prevented biofilm formation. Phage–nanocomposite conjugates (PNCs) have found their applications in the eradication of biofilms due to their ability to disturb the inner layers of biofilm structures. Finally, to target the production of bacterial biofilms, phages and nanotechnology can be used as complementing strategic methods.[93]

4.2.4 Antimicrobial photodynamic therapy (aPDT). Antimicrobial photodynamic therapies (aPDT) comprise three fundamental components. In activating the photosensitizer appropriately, a visible light source that emits a specific wavelength, a non-toxic photosensitizer (PS), and ambient oxygen that is activated to produce cytotoxic reactive oxygen species (ROS) that inactivate the targeted bacterial cells are required. Recent studies indicate that photodynamic therapy (PDT) is an innovative non-invasive treatment option that is particularly effective against superficial and localized infections caused by fungi, viruses, and bacteria that form biofilms. Antimicrobial photodynamic therapies are a novel technique with significant therapeutic potential and dental applications for treating oral infections caused by biofilms.[220–222]

4.2.5 The use of CRISPR-based gene editing to control biofilms. The CRISPR-Cas system protects bacterial cells by identifying and cleaving invasive nucleic acids. Its basic effect includes disrupting the capacity of bacteria to create several virulence factors during infection *via* controlling gene expression, development of biofilms, DNA repair, responding to stress, and recombination of resistance genes. The applications of anti-CRISPR compounds or antimicrobials against different microbes have enabled researchers to modify the CRISPR-Cas gene editing system and generate new perspectives into the functions and applications of gene editing. Zuberi *et al.*'s innovative approach of "CRISPR interference (CRISPRi)" targets a gene essential for quorum sensing, hence inhibiting the formation of bacterial biofilms. The investigation of bacterial behavioral alterations in response to varying gene expression levels is facilitated by the specific degrees of targeted knockdown produced by CRISPRi inhibition.[223] This study emphasizes the effect of the luxS gene and incorporates bacterial quorum sensing into the mix, which represents a potentially effective method for inhibiting bacterial biofilm formation and managing infections that originate from hospitals and the environment. The luxS gene is essential as it encodes the autoinducer-2 (AI-2) protein, which plays a critical role as a quorum sensing molecule in the formation and maturation stages of biofilms. The applications of CRISPRi to precisely regulate the adhesion of bacteria such as *E. coli* and prevent infections such as urinary tract infections (UTI) have also been reported.[223–225]

4.2.6 Monoclonal antibodies and biofilm eradication. The *in vivo* application of antibody-based therapy for targeting biofilms in preclinical models is limited owing to poor target specificity and infusion responses.[226] However, several studies have demonstrated the use of monoclonal antibody pools in the treatment of biofilm which decrease biofilm formation and prevent biofilm-associated infections. A typical example involved monoclonal antibodies 12C6, 12A1, and 3C1 that demonstrated inhibition of growth and attachment of biofilms to the bacterial accumulation-associated protein (AAP) when used against *S. epidermidis*.[227] The biofilms formed by

S. aureus and *P. aeruginosa* are disrupted by the native human monoclonal antibody TRL1068, which binds to DNABII proteins from both Gram-positive and Gram-negative bacteria. There have also been reports on the reduction of biofilm development when TRL1068 is combined with antibiotics. Monoclonal antibodies have been utilized in targeting specific antigens, such as adhesin proteins (ClFA, FnBPA, Can, and SasG), thereby deactivating and binding to cell wall-modifying enzymes (Atl, Atl-Amd, Atl-Gmd, and IsaA), so preventing EPS dynamic alterations. There have also been reports of monoclonal antibodies enhancing the killing effect of opsonophagocytic agents against glycopolymers such as WTA, CP, and LTA (Liu *et al.*, 2017). Positive findings have also found that employing anti-matrix component antibodies such as PNAG and DNABII, invasive proteins such as Spa, toxins such as HIa and LukAB, and proteins such as PhnD has significantly decreased the biofilm matrix.[228]

4.2.7 Vaccine developments targeting biofilms. Recently, vaccines have been developed to offer complete protection against pathogens that form biofilms. While creating vaccines for planktonic pathogens presents minimal challenges, developing vaccines that specifically target biofilms introduces a new complexity. As previously mentioned, biofilms play a crucial role in the pathogenesis of various chronic infections, and their growth enhances antibiotic tolerance and virulence while also altering metabolic activity and protein profiles.[229]

Within a biofilm, bacteria can produce two main categories of antigens, namely those that are linked to the bacterial cells forming the biofilm, and those that are part of the biofilm matrix. Antigens associated with the biofilm cells remain within the bacterial cell and are not released; in contrast, those in the biofilm matrix are secreted by the bacteria and become part of the biofilm structure. To develop vaccines that effectively target pathogens of significance, research has been directed towards identifying antigens originating from either the bacterial cells or the biofilm matrix. Vaccine development using components from the extracellular biofilm matrix is less common than using antigens obtained from biofilm cells.

These vaccine advancements include:

i. Biofilm-specific antigens: Incorporating antigens derived from biofilms into vaccine formulations is an emerging approach. These vaccines have the potential to enhance protection against infectious diseases and improve the efficacy of current vaccination methods.[230]

ii. Combination vaccines: Vaccines that combine both planktonic and biofilm antigens may offer broader protection against infections.[231]

iii. Mimicking biofilm components: The development of synthetic peptides that mimic key biofilm components can help redirect the immune response, facilitating biofilm disruption and enhancing the effectiveness of immune defenses and action of antibiotics.[232]

iv. Staphylococcal vaccine candidates: Studies are ongoing to evaluate the immunogenicity and efficacy of bacterial components involved in biofilm formation, aiming to develop effective vaccines against *Staphylococcus* infections.[233]

v. Immunomodulatory therapies: Certain companies are working on immune-enabling therapies and vaccines targeting bacterial infections associated with biofilms (Clarametyx Biosciences Awarded a Multi-Phased Agreement with CARB-X to Accelerate Anti-Biofilm Vaccine to Prevent Bacterial Infections, 2025). These approaches seek to enhance immune responses and improve antibiotic efficacy.[234]

4.2.8 Anti-quorum sensing (QS) strategies. Recently, certain agents have been developed that can specifically disrupt the QS system in bacteria, thereby impeding their communication and lowering the level of harm caused. These agents function by either primarily blocking the production of Autoinducer (AI) molecules, inhibiting AI detection through receptor inactivation, or through enzyme-catalyzed degradation or modification of AI molecules. These agents are known as quorum quenching (QQ) agents. Various classes of QQ enzymes and QQ inhibitors have been identified, categorized based on their substrate specificities as inactivators of signaling molecules, inactivators of signaling receptors, and inhibitors of signaling cascades.[235]

i. Inactivators of signaling molecules

These categories are designed to effectively disrupt bacterial communication both within and between species by suppressing the synthesis of, inactivating, or degrading AI signaling molecules. Research on certain bacteria, such as *P. aeruginosa*, has shown that (*z*)-5-octylidenethiazolidine-2,4-dione (TZD-C8) significantly reduces the expression of LuxI-type acyl-homoserine lactone synthases by interfering with the *Pseudomonas* signaling pathways (Quinolone Signal (PQS) and 3-oxo-C12-HSL) (Michaelis *et al.*, 2023). Other *in vitro* studies revealed the decrease in *N*-acyl homoserine lactone (AHL) synthesis in *P. aeruginosa* at subminimal growth-inhibitory concentrations of some macrolide antibiotics (erythromycin). It has also been reported that another macrolide, azithromycin, disrupts autoinducer synthesis by reducing the concentration of 3-oxo-C12-HSL and C4-HSL by 94% and 72%, respectively, and the expression of the transcriptional activator genes lasR and rhlR, as well as the two autoinducer synthase genes lasI and rhlI.[236] Various enzyme categories identified to inactivate signaling molecules include Lactonases, Acylases, and Oxidoreductases. These enzymes are found in environments where bacterial species reside.

ii. Inactivation of signaling receptors

Through computational docking and high-throughput screening (HTS), a few QS inhibitors that compete with autoinducers for receptor binding have been discovered, primarily in *P. aeruginosa*. A typical example is the isolated flavonoid naringenin that binds directly to the LasR receptor, thereby preventing LasR/RhlR DNA binding, thus minimizing biofilm formation and suppressing many other QS-related effects.[237] Similarly, many secondary metabolites from natural sources have been identified to suppress QS mechanisms, including embelin, *ortho*-vanillin, piperine, catechin, nakinadine B, and furanones. Other agents identified as able to bind to the LasR protein exhibiting antibiofilm activity against *P. aeruginosa* include sitagliptin and omarigliptin (Dipeptidase inhibitor-4 (DPI-4)), amikacin,

gentamicin, kanamycin, neomycin B, paromomycin, and netilmicin. More recently, Manson and colleagues, through screening a 25 000-compound library, identified eight new potent and effective antagonists of LasR.[238] In addition, many synthetic AHL analogues were identified to exert a competitive inhibition on autoinducer receptors in several bacteria.[239–242]

iii. Inhibition of signaling cascade

The use of targets to block the downstream response regulator AgrA in *S. aureus* utilizing many compounds, such as Azan-7, bumetanide, savrin, and staquorsin, has been discovered. They have the ability to reduce the AgrA–DNA complex formation at the P3 promoter region involved in the regulation of RNAIII transcription, thus preventing virulence gene upregulation and biofilm formation.[243–245] Several synthetic molecules have been identified as antagonists of LuxR-type proteins, including numerous autoinducer analogs. It has been shown that the TraR regulator response in *Agrobacterium tumefaciens* is inhibited by analogs of the autoinducer 3-oxo-octanoyl-homoserine lactone. Virstatin, a small molecule that inhibits the expression of *Vibrio cholerae* virulence factors, can suppress the expression of AnoR, a positive regulator of the LuxI-like synthase AnoI in *Acinetobacter nosocomialis*, resulting in reduced production of *N*-(3-hydroxy-dodecanoyl)-L-homoserine lactone (OH-dDHL). Lower levels of this compound disrupt the signaling cascade, leading to decreased biofilm formation and motility.[246]

4.2.9 Applications of -omics for studying and targeting biofilms. The emergence of the three omics technologies—Transcriptomics, Proteomics, and Metabolomics—has significantly advanced the studying and targeting of biofilms, aiding in their elimination. These technologies have greatly enhanced the ability to capture comprehensive systems-level information about biofilms. The transcriptome, which includes coding RNAs, determines the makeup of the proteome; thus, examining an organism's transcriptome offers an initial biochemical snapshot of gene regulation under specific conditions, such as the shift from planktonic to biofilm states and during drug treatment.[247] Techniques like quantitative polymerase chain reaction (qPCR) and high-throughput methods such as microarray and RNA-sequencing (RNA-Seq) enable real-time tracking of gene expression and can identify changes in gene expression in biofilm states or following exposure to compounds. Gene expression studies related to the biofilm's microbiome could pave the way for developing therapies and strategies to reduce the occurrence of drug-resistant biofilms. While the transcriptome typically reflects gene-level changes, analyzing the proteome profile offers a more comprehensive and stable view of the biological changes within an organism. Additionally, microbes can detect stress or harsh environmental conditions and adjust their protein expression to overcome these challenges. High-throughput proteomics has produced a wealth of information and is now widely used to study microbial biofilms. Understanding the protein expression of microbial biofilms will improve their identification and treatment. Although there are limited studies on host–biofilm interaction using proteomics, these studies have provided valuable insights into the complex mechanisms of increased resistance in microbial biofilms. The heightened resistance of microbial biofilms may be due to slower growth rates, protein synthesis, and metabolic activity within biofilm communities.[248] Microbial systems are ideal for metabolomics studies because they can be easily manipulated. However, the number and types of metabolites observed can vary significantly among different organisms, making the technology less generalizable and requiring organism-specific optimization. Key primary and secondary metabolites can greatly influence biofilm formation; therefore, metabolome profiling, combined with biological system modeling, can help identify pathways associated with biofilm formation. This can lead to new strategies for controlling biofilm formation and development.[249]

5 Biofilm models for assessing treatment efficacy

5.1 *In vitro* biofilm models

In vitro biofilm models are utilized to explore biofilm formation and evaluate treatment effectiveness. These models offer a simplified and cost-efficient way to study fundamental biofilm features such as nutrient availability and structural development. Nonetheless, they do not completely capture the complexity of natural biofilms, often using single bacterial species and failing to accurately replicate natural surfaces or nutritional conditions.[250]

There are two main methods for creating *in vitro* biofilm models: dynamic (open) and static (closed) systems.

5.1.1 Static *in vitro* biofilm models. Static *in vitro* biofilm models are straightforward and economical for examining initial biofilm formation and performing high-throughput screening. They enable the simultaneous assessment of multiple organisms, treatments, and other process parameters. However, their restricted nutrient supply and the buildup of waste products limit their use in long-term studies. Common examples include microtiter plates and colony biofilms.[251]

The following sections outline the most frequently used static *in vitro* methods.

i. Microtiter plate (MTP)

Microtiter plates are extensively used *in vitro* models for biofilm assays, facilitating high-throughput screening of various strains and conditions. Biofilm formation in the wells can be measured using colorimetric assays, although these may also stain non-biofilm components. MTT assays offers enhanced quantification of viable cells. MTPs are frequently employed to study initial biofilm formation in various organisms. They also support investigations into multi-species biofilms and attachment of microbes to diverse surfaces.[252–256] Although MTPs are instrumental in assessing novel treatment approaches and identifying microbial factors involved in biofilm formation,[7,257–260] they may interfere with biofilm development due to uncontrolled disturbances during culture and treatment. Modified MTPs—featuring varying well sizes and specialized plates for microscopy—have been developed to address these limitations.[261]

ii. **Calgary biofilm device (CBD)**

The Calgary Biofilm Device modifies the traditional microtiter plate assay by enabling robust biofilm formation on pegs affixed to a coverslip, which fits into the wells of the microtiter plate.[114] This design minimizes mechanical disturbance during media exchange—a key factor when cultivating older biofilms for up to 12 days. The primary advantage of this device is its ability to isolate and measure sessile cell biomass exclusively, offering a more accurate assessment of biofilm mass than methods that include planktonic cells.[261]

iii. Biofilm ring test (BRT).

The Biofilm Ring Test evaluates early biofilm formation by measuring the immobilization of magnetic beads by bacteria. In this closed model, beads are mixed with bacterial suspensions in microtiter wells. When exposed to a magnetic field, free beads move, but their movement is increasingly restricted as biofilm forms. This allows for real-time analysis at multiple intervals.[262] Surgers *et al.* used the BRT to study biofilms from ESBL-producing *E. coli* and *K. pneumoniae*.[263] Unlike traditional assays, BRT requires no staining or fixation, reducing bias and enabling rapid results. However, like other 2D models, it does not reveal structural biofilm details and is affected by sedimented cells.[264]

5.1.2 Dynamic *in vitro* models of biofilm. Dynamic *in vitro* models simulate shear forces by pushing liquid through a system or by adding glass beads to rotating microtiter wells. These setups replicate the flow dynamics seen *in vivo* and allow for detailed study of biofilm development and antimicrobial resistance. They are particularly valuable in wound research due to their ability to mimic fluid flow from blood vessels. These open systems support continuous nutrient delivery and waste removal, enabling prolonged cultivation of mature biofilms. However, they are complex, requiring specialized skills and equipment, and are not suitable for high-throughput screening due to their cost and setup demands.[253]

Common dynamic *in vitro* biofilm models are discussed in the following sections.

i. Modified Robbin's device (MRD)

The original Robbins device was designed to monitor biofilm formation in simulated drinking water systems with variable flow.[265] The modified version, adapted for small-scale laboratories, supports biofilm growth on removable coupons or slides under controlled flow conditions. The MRD features a pipe with holes for placing coupons in a liquid stream, with adjustable flow speeds. Coupons are aligned parallel to the flow and can be removed for analysis. This model is widely used to assess biofilm formation under various hydrodynamic conditions and can sustain biofilms for weeks.

However, flow must be fully developed at the coupon site to avoid entry effects and ensure reliable comparisons. The model's limitations include low throughput, high cost, and potential artefacts during coupon removal.[264]

ii. Drip flow reactor (DFR)

The drip flow reactor supports biofilm formation under low shear stress in a continuous flow environment. It consists of tilted parallel channels with inserted coupons or slides. During operation, the system tilts approximately 10 °C from horizontal, allowing a slow flow of fluid along the coupons and creating low shear conditions. Advantages include operational simplicity, the ability to test various surfaces concurrently, and opportunities for non-invasive analysis.[264] This model is useful for studying biofilm heterogeneity under low shear.

iii. Rotary reactors

There are three main types of rotary reactors used for biofilm formation: rotary annular, rotary disk, and concentric cylinder reactors.[264] Rotary annular reactors consist of a stationary outer cylinder and a rotating inner cylinder controlled by a motor to maintain constant shear stress. Rotary disk reactors use a rotating disk powered by a magnetic stirrer, with coupons placed at varying radial positions to expose them to different shear stresses. Concentric cylinder reactors feature four chambers, each with its own rotating cylinder, allowing the simultaneous testing of different shear conditions. These reactors allow the examination of up to four bacterial strains per experiment, though they limit sampling to one surface. In rotary disk reactors, shear stress and flow rate can be adjusted independently.[265] Despite these capabilities, rotary reactors are not suitable for high-throughput applications.

5.2 3D-biofilm techniques

3D printing models enable the construction of biofilm models that closely replicate the architecture of *in vivo* biofilms, offering more realistic simulations than traditional 2D models. 3D bioprinting employs biomaterials or living cells to create biofilm structures. Bioinks—polymer solutions embedded with bacterial cells—form gels in response to stimuli.[114] Natural polymers like gelatin and alginate provide biocompatibility, while synthetic polymers like polyethylene glycol enhance versatility. This technique enables evaluation of antimicrobial penetration through biofilms with controlled thickness and geometry. Ning *et al.* developed alginate-based biofilms exceeding 4 mm in thickness, which exhibited enhanced resistance to antimicrobials.[266] 3D printing also supports studies on biofilm surface properties and resistance mechanisms.[267,268]

5.3 Microcosm biofilm model system

Combating antibiotic resistance remains a major challenge in microbiology and medicine. Understanding microbial biofilms is essential for developing new therapeutic strategies.[269] The microcosm biofilm model closely mimics *in vivo* conditions, offering a robust *in vitro* platform for studying biofilm behavior.[270] Several *in vitro* models have been established to evaluate treatment efficacy (Table 2).

6 Future prospects for biofilm eradication

The field of microbial biofilms has grown significantly over the last two decades, which has served to shed light on the

Table 2 Summary of different biofilm models, key features, advantages and limitations

Model type	Model	Key features	Advantages	Limitations
Static *in vitro* models	Microtiter Plate (MTP)	2D wells for biofilm growth; quantification *via* colorimetric/MTT assays	High-throughput; simple; supports multi-species and surface studies	May disrupt biofilms; stains non-biofilm matter; limited structural info
	Calgary Biofilm Device (CBD)	Pegs allow undisturbed biofilm growth; separate sessile biomass	Minimizes disturbance; supports long-term growth; accurate biofilm quantification	Specialized setup; still limited to static environments
	Biofilm Ring Test (BRT)	Uses magnetic beads to track early biofilm immobilization	Real-time, rapid results; no staining/fixation needed	Doesn't assess structure; 2D only; sedimented cells may affect accuracy
Dynamic *in vitro* models	Modified Robbin's Device (MRD)	Flow-through system with removable coupons	Simulates hydrodynamic conditions; long-term growth; evaluates surfaces	Artefacts from coupon removal; low throughput; costly
	Drip Flow Reactor (DFR)	Low shear flow on tilted channels with slides/coupons	Easy to use; allows different surfaces; non-invasive analysis	Simulates only low shear; limited control over flow dynamics
	Rotary reactors	Rotating components generate shear; test multiple shear levels simultaneously	Good shear control; assess resistance and structure under varied conditions	Low throughput; complex setup; limited sampling (esp. in concentric model)
Advanced models	3D-biofilm techniques	Uses 3D printing/bioprinting with bioinks and various polymers	Mimics 3D structure; custom dimensions; studies penetration and surface resistance	Requires expertise; costly; still developing
Microcosm model	Microcosm biofilm model	Simulates natural/mixed- species biofilms under near-physiological conditions	Closely resembles *in vivo* settings; reliable for antimicrobial evaluation	Less standardization; potential variability

phenomenon's complexity. However, biofilm-linked infections still represent a serious health risk due to their persistence. Therefore, deliberate efforts are required to advance our understanding of the structure, composition, physiology, dynamics and genetics of bacterial biofilms—as they pertain to chronic diseases and infections. Further studies ought to identify the genes encoding each stage in the biofilm formation, including those essential for the initial transformation of planktonic cells into sessile forms, and the various mechanisms by which biofilms develop resistance to microbes. The combination of developments in transcriptomics, metabolomics, and transposon-based next-generation sequencing will be of great help in the identification of new genetic targets for biofilm research.[138] It should also include research on how to counteract antimicrobial resistance. Disciplines relating to microbes have acknowledged the ubiquitous nature of the biofilm phenotype and so researchers in such disciplines should concentrate on the threat posed by biofilms in order to gain a deeper understanding of their colonization processes. The implementation of this strategy by the pharmaceutical and healthcare sectors will undoubtedly result in the creation of novel methods for the prevention and control of infections. A clearer understanding of the factors that separate the sessile phenotype from the planktonic phenotype will be essential for the effectiveness of any future controls on biofilms (Table 3).[281]

All of the natural anti-biofilm techniques that have been presented are important research areas; unfortunately, they are still in the early stages of development and have not yet completed the clinical trial stages, and hence are not yet on the open market. Future studies on biofilm infection prevention and management approaches ought to concentrate on various prophylactic and corrective actions against biofilm formation and colonization of medical devices. To improve the anti-biofilm efficacy of natural agents, combination therapy with conventional antibiotics needs to be explored as a prospect. Natural quorum quenchers have a prospective application in the biomedical industries and can be a novel lead for species-specific biofilm eradication when combined with antibiotics. Further research in this area is necessary to turn the innovative anti-biofilm phytocompounds into pharmaceuticals.[282]

Also, the combination of antibiofilm agents with nanoparticles (nanoparticle-based antibiofilm agents) has demonstrated encouraging outcomes in the fight against bacterial biofilms. Novel minute antimicrobial peptides could lead to improved treatment of respiratory infections linked to biofilms. Potential therapeutic applications for these peptides will be facilitated by an understanding of the molecular pathways *via* which they alter host immunity, pathogenicity, and biofilm signaling.[283]

The evaluation of the concurrent use of biofilm-targeted immunotherapy and anti-biofilm agents with different mechanisms of action is also necessary. This approach has the potential to (i) disperse biofilms, break down extracellular polymers (EPSs), and get rid of persister cells all at once, thereby significantly increasing the potential for the successful clinical eradication of established biofilms, and (ii) combat the emergence of antimicrobial resistance brought about by the usage of individual antibiotics. Furthermore, approaches are required to prevent wrong and excessive dosages which promote cytotoxicity to the host and antimicrobial resistance in the biofilms. In such designs, it is imperative to design safe, effective and cost-effective antibiofilm drug delivery methods. To maximize therapeutic efficacy and specificity to the biofilm matrix, drug delivery methods can target specific bacterial components (*e.g.*, lipopolysaccharides) or metabolites (*e.g.*, endotoxins).[284]

Even with recent advances in biofilm research, further study is necessary to guarantee their effective and safe use in

Table 3 Developed microcosm biofilm model for assessing treatment efficacy

S/N	Biofilm model	Treatment	Result	Ref.
1	Layered chronic wound biofilm (CWB)	Antimicrobial solution and dressings (containing silver or honey)	Moderate-to-low antibiofilm effect. The model exhibits resilience to antibiofilm treatment.	271
2	Multi species oral biofilm model	Miconazole (MCZ) nanocarrier-based delivery system	MCZ nanocarrier-based delivery system was more effective than MCZ alone.	272
3	Supplemented oral biofilm model	Iron oxide nanoparticles (IONPs) coated with chitosan (IONPs-CS) as carriers of miconazole (MCZ) or fluconazole (FLZ)	The use of nanocarriers was effective against oral fungal infections.	273
4	Oral biofilm model	Essential oils (*Cymbopogon citratus* extracts). Chlorhexidine digluconate	The combination of *C. citratus* and chlorhexidine digluconate decreased the microbial viability of biofilms.	274
5	Oral biofilms on bovine tooth specimens	Two concentrations (0.025 mg ml^{-1}, and 0.05 mg ml^{-1}) of Sugarcane cystatin (CaneCPI-5)	CaneCPI-5 did not alter the microcosm biofilm viability.	275
6	Bovine tooth model	A new photosensitizer named Fotoenticine® (FTC) derived from chlorine e-6.	FTC was efficacious against the heterogeneous biofilms of dental caries.	276
7	Hydroxyapatite disks biofilm model	Chlorhexidine gluconate (CHX; 0.12%) and Cold Atmospheric Plasma (CAP)	CAP was more effective in reducing oral microcosm biofilm pathogenicity than 0.12% CHX	277
8	Amsterdam Active Attachment model (AAA-model)			278
9	Human plasma biofilm model (hpBIOM)	Antiseptics (Octenisept® and Lavasorb®)	Efficacy in the hpBIOM	279
10	Wetland biofilm model	Glyphosate pesticide	Glyphosate and its degradation products bioaccumulate in the microcosms and reduce the algal components of the biofilm	280

therapeutic environments. A coordinated effort across various disciplines is needed to translate complex antibiofilm medications from controlled *in vitro* or *in vivo*-like conditions into real-world clinical settings. All researchers in related disciplines have pivotal roles to play in propelling this crucial initiative forward, bringing the healthcare discipline closer to more effective solutions for the management of biofilm-associated infections.[285]

The following areas will be the primary focus of the development trend for controlling biofilms in the future: (a) Analysis of the architectural make-up of biofilms; controlling the formation of biofilms requires a thorough understanding of their composition and structure. Subsequent investigations will concentrate on delving into insights at the molecular level and utilizing surface nanotechnology to enhance comprehension and address the bacterial biofilm development process. (b) Research and use of new sterilization techniques: future studies should investigate and implement novel sterilizing techniques and methods in nanotechnology and bioengineering, in addition to conventional antibacterial medications. (c) Creation of new antimicrobials: presently, a growing number of microorganisms are developing resistance to conventional antibiotics.

To effectively tackle bacterial biofilms, future research should concentrate on creating new synthetic or natural antibiotics and antimicrobials. (d) Even though biofilms are regarded as pathogens, they can be a serious threat to several other industries, hence the need to pay close attention to biofilms in other areas. For instance, the formation and adhesion of biofilms on food and food processing equipment increases the risks and burdens associated with food safety and health. Additionally, biofilms in sewage present obstacles to wastewater treatment procedures. Also, there has been increased growth of biofilms in veterinary systems. Future investigations and research on managing biofilms in several domains ought to be of interest.[135]

6.1 Conclusion

Biofilms present a formidable challenge in many clinical, environmental, and industrial processes through their complex structure, resilience, and resistance mechanisms. This review uncovers their structure, formation, resistance mechanisms, and novel and emerging advances in antibiofilm research. Specifically, it elucidates the promising strides emanating from the use of therapeutic natural products, adjuvants, nanotechnologies, photodynamic therapy, gene editing techniques, monoclonal antibodies, vaccine developments, anti-quorum sensing (QS) strategies, applications of -omics for studying and targeting biofilms and biofilm eradication models. Nevertheless, these promising advances and technologies are still in the bench stages and needing translation to clinical practices. Thus, the clarion call is for multidisciplinary efforts including the optimization of the emerging options, evaluation of the delivery profile and administration routes, and extensive preclinical and clinical trials.

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Conflicts of interest

All authors have approved the final review manuscript and all declare they have no conflict of interest.

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No primary research results, software or code have been included and no new data were generated or analyzed as part of this review.

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