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Strategies for Detecting Bacterial Biofilms: Unveiling the Hidden World of Microbial Aggregates

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THE DETECTION of bacterial biofilms is essential for understanding their role in various fields, including medicine, industry, and environmental science. Traditional culture-based methods, despite their widespread use, have limitations in estimating biofilm biomass and providing comprehensive species composition information. Physical methods, such as direct observation and microscopy techniques, offer high-resolution visualization but may lack sensitivity. Chemical methods, including staining techniques and ATP-based assays, provide simple insights but face challenges in differentiating live and dead cells. Immunological methods, such as ELISA and immunofluorescence microscopy, offer high specificity but can be expensive and require expertise. Molecular methods like PCR, qPCR, and FISH enable specific detection but require optimization and cannot distinguish live from dead cells. Emerging technologies, such as biosensors, acoustic techniques, and mass spectrometry, hold promise for rapid, real-time monitoring but require further validation. Moving forward, considerations such as multimodal approaches, in situ detection methods, species-specific targeting, and automation and miniaturization will be crucial for advancing biofilm detection capabilities. By embracing these future directions, researchers can enhance our understanding of biofilm biology and develop targeted strategies for combating biofilm-related infections and environmental contamination.

Keywords: Biofilm, Chemical method, Immunological method, Microbes, Molecular method, Soil.

Introduction

Bacterial biofilms are complex communities of microorganisms encased in a self-produced extracellular polymeric substance (EPS) matrix (Maier, 2021; Wong et al., 2021). Biofilm formation is marked by the irreversible attachment of microbial cells to surfaces or each other, where they become embedded in extracellular polymeric substances (EPS) and display distinct phenotypes regarding gene transcription and growth rates. These biofilms can consist of a single microorganism or a diverse mixture of bacteria, fungi, archaea, protozoa, and yeasts. Additionally, biofilms often possess a channel-like structure that regulates the release of gases, nutrients, and antimicrobial agents (Zhao et al., 2023). These biofilms are ubiquitous in nature and can colonize diverse environments, ranging from natural habitats like soil, water, and rocks to artificial surfaces such as medical devices, industrial pipelines, and food processing equipment (Schulze et al., 2021; Lili et al., 2023). Almost all bacteria have the potential to form biofilms under certain conditions (Zhao et al., 2023). The ability of bacteria to form biofilms is a fundamental survival strategy, enabling them to persist in harsh conditions, resist antimicrobial agents, and establish persistent infections. Microorganisms that are capable of producing biofilms contribute significantly to nosocomial and recurrent infections. The main virulence factor in biofilm-related infections is the sticky exopolysaccharide matrix constituting the biofilm. The process of biofilm formation is intricate and involves various developmental phases, some of which are unique to the specific type of bacteria involved. The process of biofilm formation can be distributed into four phases, i) initiated with bacterial attachment to surfaces, which can be biotic, such as host cells, or abiotic, such as prosthetic devices. Following attachment, ii) bacterial aggregation through cell-cell adhesion is the initial step leading to the formation of the biofilm structure, facilitated by adhesin molecules on the bacterial cell surface (Costerton et al., 1999). Some bacteria utilize fimbriae and flagella for initial attachment (Toutain et al., 2007). Following successful adhesion, iii) bacterial cells divide and produce extracellular polymeric substances (EPS), which promote irreversible attachment and create a

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complex matrix (Stoodely et al., 2002). This highly hydrated EPS, composed of polysaccharides, proteins, lipids, and extracellular or exogenous DNA (eDNA) (Chiba et al., 2022; Schilcher and Horswill, 2020), accounts for a significant portion of the biofilm's mass, estimated at around 90% (Flemming and Wingender, 2010). It is generally believed that biofilm matures after 24 h and forms a thick layer of biomolecules. iv) The final phase of bacterial biofilm development involves dispersal, which encompasses several mechanisms unique to different bacteria. As biofilms reach maturity, dispersal becomes a viable choice. However, these process typically include three primary processes: detachment of bacterial cells from small colonies, transfer of cells to alternate substrates, and attachment to new substrates (Shen et al., 2018). The detachment process may be either active or passive in nature (Zhao et al., 2023). Several factors can trigger the detachment of biofilm cells, including changes in nutrient availability, fluctuations in oxygen levels, accumulation of toxic products, and other stress-inducing conditions (Sauer et al., 2004; Karatan and Watnick, 2009; Hong et al., 2010; Rowe et al., 2010; Mosad et al., 2024). Within a multi-layered biofilm, cells undergo cell-to-cell interactions, occurring both within the biofilms themselves, in direct contact with the solid surface, and within flocs, where mobile biofilms form without adhering to a surface. These biofilms represent a significant challenge in various fields, including healthcare, industry, and environmental management. These complex microbial communities adhere to surfaces and form structured matrices, providing protection and resilience against external threats such as antibiotics (Stewart and Costerton, 2001), disinfectants, and host immune responses. Consequently, biofilm-related infections pose a significant threat to human health, contributing to diagnosis challenging (Roy et al., 2018). Therefore, there is an urgent need for sensitive and reliable methods to detect and monitor bacterial biofilms in various contexts. Early and accurate detection of biofilms is crucial for effective treatment and prevention management strategies of biofilm-related infections. However, their heterogeneity, hidden nature, and diverse environments present significant obstacles. Recent advances in detection techniques have expanded our ability to visualize, characterize, and monitor biofilms with high sensitivity and resolution. By targeting different aspects of biofilm biology, these techniques provide valuable insights into biofilm structure, composition, and behaviour, the persistence and recurrence of chronic diseases, complicating medical treatments, and increasing healthcare costs.

Detection and characterization of bacterial biofilms are critical for understanding their role in infections, biofouling, and biocorrosion, as well as for developing effective strategies to control their formation and dispersal (Funari and Shen, 2022). Dispersion of biofilm can be triggered by various conditions, such as the presence of phenol-soluble modulins (PSMs), prompting sessile bacteria to revert to a planktonic state (Peng et al., 2022; Kırmusaoğlu, 2019). Bacteria residing within the biofilm (sessile form) exhibit greater resistance to antimicrobial agents compared to their planktonic counterparts, rendering treatment of biofilm-embedded bacteria challenging (Schulze et al., 2021). In recent years, significant progress has been made in developing innovative techniques for detecting and visualizing bacterial biofilms. These techniques leverage advances in molecular biology, microscopy, imaging, and sensor technology to overcome the limitations of conventional methods and provide insights into biofilm structure, composition, and behaviour. By targeting specific biomolecules, cellular structures, or metabolic activities associated with biofilm formation, these methods offer high sensitivity, specificity, and resolution for biofilm detection in diverse environments. One of the key challenges in managing biofilm-related infections is the difficulty in detecting and diagnosing biofilm presence in clinical settings (Mendhe et al., 2023). Traditional microbiological methods, such as culture-based techniques, often fail to detect biofilms due to their sessile nature and altered phenotype compared to planktonic bacteria. Moreover, biofilm-associated infections are frequently asymptomatic or present with nonspecific clinical symptoms, making paving the way for improved diagnosis, treatment, and prevention of biofilm-related infections and environmental issues.

In this review, we will comprehensively explore current strategies for detecting bacterial biofilms, highlighting their principles, advantages, limitations, and potential applicationsadditives.

2. Strategies for Identification of Biofilms

Detection of bacterial biofilms involves two main approaches: targeting extracellular polymeric substances (EPS) or specific microbial biomarkers/genetic signatures. EPS, comprising polysaccharides, proteins, and eDNA, forms the biofilm matrix, crucial for adhesion, cohesion, and

protection (Di Martino, 2018). Techniques like lectin staining, fluorescent probes, and Fourier-transform infrared spectroscopy (FTIR) visualize and quantify EPS, providing insights into biofilm architecture and composition (Cattò and Cappitelli, 2019; Wang et al., 2022). Molecular methods, such as fluorescent in situ hybridization (FISH), quantitative polymerase chain reaction (qPCR), and next-generation sequencing (NGS), target microbial biomarkers or genes, aiding in identifying biofilm-associated species and understanding community dynamics (Barbosa et al., 2023). Advanced imaging modalities, like confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM), offer high-resolution visualization of biofilm structure and dynamics (Relucenti et al., 2021). Live-cell imaging enables real-time monitoring of biofilm formation and behaviour. Comprehensively understanding these techniques' principles, advantages, and limitations is essential for developing effective biofilm detection strategies with applications across healthcare, industry, and environmental management (Fig. 1).

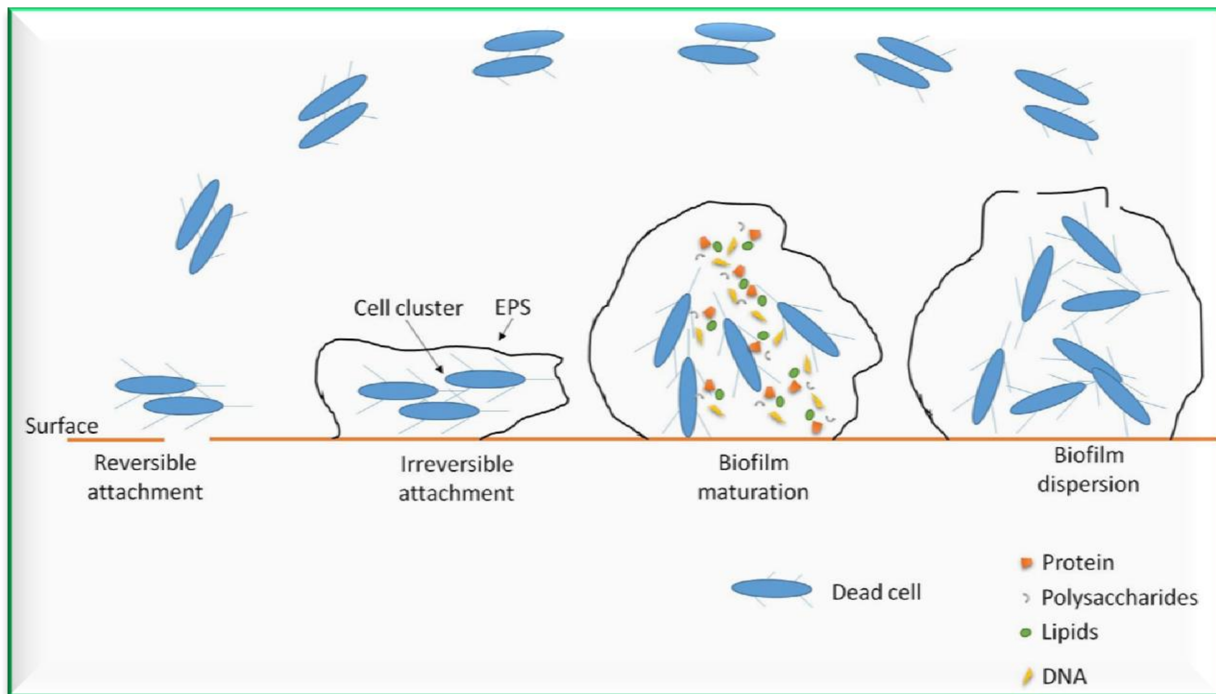

Fig. 1. Different stages of biofilm formation through bacteria.

2.1 Traditional culture-based methods

2.1.1 Direct plating

For decades, culture-based methods have served as the cornerstone in identifying bacterial biofilms, playing a crucial role in understanding their composition and unravelling their complexities. These techniques, although established, offer valuable insights into the diverse microbial communities that form biofilms and unveil the specific bacterial culprits involved. Culture-based methods rely on the fundamental principle of growing bacteria in a controlled environment that mimics their natural habitat. For biofilm analysis, samples are collected from the suspected location, such as medical devices, catheters, or environmental surfaces. A sample containing the biofilm is collected and homogenized to dislodge the embedded bacteria. This homogenate is then plated onto media, allowing individual bacterial colonies to grow. After incubation, these colonies can be identified visually based on their morphology and colony characteristics, such as colour, size, and texture. Before cultivation can occur, the biofilm's protective EPS matrix needs to be disrupted. This is often achieved through various techniques, such as sonication, which utilizes sound waves to dislodge embedded cells, or enzymatic treatments that break down specific components of the EPS. This initial step allows for the release of viable bacterial cells, making them accessible for further analysis. While seemingly simple, this method has limitations. First, it may not capture the full diversity of the biofilm community, as certain bacterial species might require specific growth conditions or may not be readily culturable (Bjarnsholt, 2013). Additionally, distinguishing between viable and non-viable cells

within a colony can be challenging (Darvishi et al., 2022).

2.1.2. Cultivation on selective media

To overcome the limitations of direct plating, enrichment techniques are often employed. These involve culturing the biofilm sample in liquid media specifically designed to favour the growth of desired bacterial groups (Darvishi et al., 2022). This selective pressure allows for the enrichment of specific bacterial populations that might be present in lower numbers within the biofilm, making them easier to detect and identify subsequently. This can be particularly relevant for uncovering fastidious or slow-growing bacterial species that might be outcompeted on general media (Sehar and Naz, 2016).

Once the biofilm is disrupted, the released cells are inoculated onto specific culture media. These media are formulated with various nutrients and selective agents that favour the growth of specific bacterial species while inhibiting the growth of unwanted microorganisms. For example, blood agar may be used to identify potential pathogens, while specific media like mannitol salt agar might be used to isolate *Staphylococcus aureus* (Manandhar et al., 2018). After incubation under controlled conditions of temperature and atmosphere, colonies of different bacterial species emerge, allowing for their preliminary identification based on their colony morphology and growth characteristics (Coraça-Huber et al., 2020).

2.1.3. Identification techniques

Once potential biofilm-forming bacteria are isolated through direct plating or enrichment techniques, further confirmation of their identity is essential. This is typically achieved through a combination of phenotypic and genotypic tests. Phenotypic tests involve analyzing various observable characteristics of the isolated bacteria, such as their ability to utilize specific sugars (Khochamit et al., 2015), produce certain enzymes (Bascomb and Manafi, 1998), or resist specific antibiotics (Qi et al., 2006). Following the initial selection based on colony appearance, further identification steps are undertaken. These may involve: i) Gram staining: This simple yet effective stain differentiates bacteria based on their cell wall structure, providing a broad classification into Gram-positive and Gram-negative groups. ii) Biochemical tests: Specific tests are employed to confirm the identity of suspected bacterial species. These tests leverage the bacteria's ability to utilize different substrates or produce specific enzymes, allowing for definitive identification. iii) Antibiotic susceptibility testing: This crucial step assesses the susceptibility of isolated bacterial strains to various antibiotics, providing valuable information for devising effective treatment strategies against biofilm-related infections (Table 1).

Traditional culture-based methods offer several advantages. They are relatively inexpensive, widely available, and provide valuable information not only on the identity of bacterial species but also their culturability and antibiotic susceptibility (Zhang et al., 2021a). However, limitations also exist. These methods can be time-consuming, requiring several days to obtain results. Additionally, they may not effectively capture the full diversity of bacterial communities within a biofilm, as some species might be slow-growing or fastidious, requiring specialized media or conditions for their growth (Srinivasan and Fredricks, 2008). Despite these limitations, culture-based methods remain crucial tools in the biofilm research arsenal, offering valuable information for understanding the composition and dynamics of these microbial communities.

Table 1. Strategies for biofilm identification.

S.N.	Strategies for identification of biofilms		References
1	Traditional culture-based methods	Direct plating	Wang et al. (2022)
		Cultivation on selective media	Darvishi et al. (2022)
		Identification techniques	Qi et al. (2006)
2	Physical method	Direct observation	Pereira et al. (2022)
		Microscopy techniques	Mhade and Kaushik (2023)
3	Chemical methods	Staining techniques	Wilson et al., 2017
		ATP-based assays	Lee et al., 2010
4	Immunological methods	Enzyme-linked immunosorbent assay (ELISA) based techniques	Singh et al. (2021)
		Immunofluorescence microscopy	Shakes et al. (2012)
5	Molecular methods	Polymerase chain reaction (PCR) based	Millar et al. (2007)
		Fluorescence in situ hybridization (FISH)	Strelkova et al. (2013)
6	Emerging technologies	Biosensor	Pu et al. (2021)
		Acoustic techniques	Subramanian et al. (2020)
		Mass spectrometry	Sportelli et al. (2022)

2.2. Physical method

2.2.1. Direct observation

Visual inspection and light microscopy play integral roles in detecting bacterial biofilms, providing researchers with valuable insights into biofilm structure, composition, and spatial organization (Valdivieso González et al., 2023; Pereira et al., 2022). This methodological approach, although relatively straightforward, offers significant advantages in biofilm analysis due to its accessibility, versatility, and ability to provide qualitative and quantitative information. Visual inspection serves as the initial step in biofilm detection, allowing researchers to identify potential biofilm formation on various surfaces or substrates (Pereira et al., 2022). This process involves the naked-eye examination of surfaces for visual cues such as discoloration, slime formation, or irregular surface morphology, which are indicative of biofilm presence (Relucenti et al., 2021). While visual inspection alone may not provide definitive evidence of biofilm formation, it serves as a rapid screening tool to identify areas for further analysis using more specialized techniques such as light microscopy.

Light microscopy represents a powerful tool for characterizing bacterial biofilms at the microscale level, enabling researchers to visualize biofilm architecture, microbial distribution, and cellular morphology with high resolution (Relucenti et al., 2021). Various microscopy techniques, including bright-field, phase-contrast, and fluorescence microscopy, offer distinct advantages for biofilm analysis, allowing researchers to probe different aspects of biofilm structure and dynamics. Bright-field microscopy, the most commonly used technique in biofilm research, provides detailed images of biofilm morphology and structure by illuminating the sample with a broad spectrum of light (Relucenti et al., 2021). Additionally, bright-field microscopy enables researchers to detect biofilm-associated features such as microcolonies, extracellular polymeric substances (EPS), and water channels, which are critical for biofilm development and function (Wilson et al., 2017). These techniques allow researchers to observe

In addition to qualitative analysis, light microscopy can be coupled with image analysis software to perform quantitative measurements of biofilm parameters such as biofilm thickness, surface coverage, and cellular arrangements, providing valuable qualitative information about biofilm architecture. Digital image processing algorithms enable investigators to extract valuable quantitative data from microscopy images, providing insights into biofilm growth kinetics, microbial activity, and structural heterogeneity (Achinas et al., 2020). Phase-contrast microscopy enhances the contrast of transparent samples such as bacterial cells and EPS, allowing investigators to visualize biofilm architecture with greater clarity and detail (Wilson et al., 2017). By exploiting differences in refractive index between cellular and non-cellular components of the biofilm, phase-contrast microscopy enables the visualization of fine structural details such as cell morphology, EPS matrix, and biofilm interfaces. This technique is particularly useful for studying biofilm dynamics and interactions between microbial cells and their environment. Fluorescence microscopy offers unparalleled sensitivity and specificity for visualizing biofilm-associated components such as cells, EPS, and extracellular DNA (eDNA) using fluorescently labeled probes or stains. It allows the scientists to distinguish between live and dead cells within the biofilm, assess microbial viability, and quantify biomass distribution. Moreover, fluorescence microscopy enables the visualization of specific microbial populations within the biofilm using fluorescently labeled antibodies or nucleic acid probes, facilitating the study of biofilm heterogeneity and community structure (Sugimoto and Kinjo, 2023; Wang et al., 2024). Furthermore, advances in automated microscopy systems allow for high-throughput screening of biofilm samples, enabling rapid analysis of large sample sets and facilitating comparative studies across different experimental conditions.

Overall, visual inspection and light microscopy represent indispensable tools for detecting bacterial biofilms and unravelling their complex architecture and dynamics. By combining qualitative and quantitative approaches, researchers can gain a comprehensive understanding of biofilm formation, growth, and behaviour, paving the way for the development of novel strategies for biofilm control and management in diverse biomedical, environmental, and industrial settings.

2.2.2. Microscopy techniques

Detecting bacterial biofilms through physical methods involves various techniques aimed at visualizing and characterizing these complex microbial communities on surfaces or within host environments. These methods play a crucial role in understanding biofilm formation, assessing biofilm-related infections, and developing strategies for biofilm control and eradication. One common physical method for detecting bacterial biofilms is microscopy, which provides high-resolution imaging of biofilm structure and morphology. Confocal laser scanning microscopy (CLSM) allows for three-dimensional visualization of biofilm architecture, revealing spatial organization, microbial distribution, and extracellular matrix components (Schlafer and Meyer, 2017; Mhade and Kaushik, 2023). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) offer detailed ultrastructural analysis of biofilm composition, including microbial cells, extracellular polymeric substances (EPS), and surface interactions

(Huang et al., 2022; Cleaver and Garnett, 2023; McCutcheon and Southam, 2018). Atomic force microscopy (AFM) enables nanoscale imaging and mechanical mapping of biofilm surfaces, elucidating adhesive forces, topographical features, and cell-substrate interactions (Dufrêne, 2014; Wright et al., 2010) (Table 2).

Table 2. Microscopy techniques.

S.N.	Microscopic techniques	Work	References
1	Confocal laser scanning microscopy (CLSM)	Allows for three-dimensional visualization of biofilm architecture, revealing spatial organization, microbial distribution, and extracellular matrix components	Schlafer and Meyer (2017)
2	Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)	Offer detailed ultrastructural analysis of biofilm composition, including microbial cells	Huang et al. (2022)
3	Extracellular polymeric substances (EPS)	Surface interactions	Cleaver and Garnett (2023)
4	Atomic force microscopy (AFM)	Enables nanoscale imaging and mechanical mapping of biofilm surfaces, elucidating adhesive forces, topographical features, and cell-substrate interactions	Dufrêne (2014)

2.2.3. Spectroscopic techniques

Apart from microscopy, physical methods for detecting bacterial biofilms include spectroscopic techniques such as Fourier-transform infrared (FTIR) spectroscopy, Raman spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy (Nag et al., 2021; Zhang et al., 2021b). These methods provide chemical fingerprinting of biofilm components, identifying biomolecules, and characterizing metabolic activities within biofilm communities (Franco-Duarte et al., 2019). FTIR spectroscopy elucidates molecular composition and structural changes in biofilm samples, while Raman spectroscopy offers label-free identification of microbial species and metabolites (Parmar et al., 2024; Keleştemur et al., 2018). NMR spectroscopy provides insights into biofilm metabolism, nutrient utilization, and metabolic pathways, facilitating metabolic profiling and biomarker discovery (Leggett al., 2022). Moreover, physical methods for detecting bacterial biofilms encompass rheological and mechanical approaches, including rheometry, microfluidics, and quartz crystal microbalance (QCM). Rheometry measures the viscoelastic properties of biofilms, assessing their mechanical strength, stability, and response to shear forces (Gloag et al., 2019; Boudarel et al., 2018). Microfluidic devices enable controlled manipulation and observation of biofilm growth under dynamic flow conditions, simulating natural environments and facilitating real-time monitoring of biofilm development (Kim et al., 2012). QCM quantifies mass changes and viscoelastic properties of biofilm-covered surfaces, detecting bacterial adhesion, biofilm formation, and antimicrobial efficacy (Azeredo et al., 2017).

Furthermore, physical methods for detecting bacterial biofilms encompass surface analysis techniques such as surface plasmon resonance (SPR), ellipsometry, and profilometry. SPR measures changes in refractive index upon biofilm formation, providing real-time monitoring of bacterial adhesion and biofilm growth on sensor surfaces. Ellipsometry quantifies changes in surface thickness and refractive index, elucidating biofilm structure, growth kinetics, and antimicrobial interactions (Höök et al., 2001). Profilometry characterizes surface roughness and topography, assessing biofilm attachment, surface coverage, and substrate modifications (Teutle-Coyotecatl et al., 2022). Ultrasound imaging enables non-invasive visualization of biofilm presence, distribution, and thickness in soft tissues and biomaterials (Vaidya et al., 2014). Magnetic resonance imaging (MRI) provides high-resolution imaging of biofilm-associated infections *in vivo*, offering insights into biofilm spatial distribution, host-pathogen interactions, and treatment responses (Herrling et al., 2019). Optical coherence tomography (OCT) enables cross-sectional imaging of biofilm structure and thickness in transparent materials, facilitating real-time monitoring of biofilm growth and eradication in biomedical and industrial settings (Nguyen et al., 2013).

Physical methods for detecting bacterial biofilms encompass a diverse array of techniques ranging from microscopy and spectroscopy to rheology, surface analysis, and imaging modalities. These methods offer valuable insights into biofilm structure, composition, mechanics, and dynamics, enhancing our understanding of biofilm-related phenomena and informing the development of novel diagnostics, therapeutics, and preventive strategies

for biofilm-associated infections and biofouling phenomena in various biomedical, environmental, and industrial contexts.

2.3. Chemical methods

2.3.1. Staining techniques

Detecting bacterial biofilms is crucial for various fields, including medicine, industry, and environmental science, where these complex microbial communities pose significant challenges. Staining methods play a pivotal role in visualizing and characterizing biofilms, offering insights into their structure, composition, and metabolic activity (Wilson et al., 2017). Among the staining techniques employed, crystal violet (Vazquez et al., 2020), Propidium Iodide (PI; Vazquez et al., 2020), SYTO 9 (Vazquez et al., 2020), Fei-Mao (FM) dyes (Johnson et al., 2013), DAPI (4′,6-Diamidino-2-phenylindole dilactate; Chimileski et al., 2014) and Calcein (Tsai et al., 2013) stand out as effective tools for distinguishing biomass and metabolically active cells within biofilms (Wilson et al., 2017).

Crystal violet staining is a classic method widely used for biofilm detection and quantification. This technique involves the application of a crystal violet solution to biofilm-covered surfaces, followed by rinsing and destaining to remove excess dye. The dye binds to the extracellular polymeric substances (EPS) and bacterial cells within the biofilm matrix, staining them purple or blue. After destaining, the intensity of the stain is measured spectrophotometrically or visually, providing a quantitative assessment of biofilm formation (Wilson et al., 2017; Yaseen and Yossif, 2019). One example of crystal violet staining application is in medical settings, particularly in diagnosing bacterial infections associated with biofilm formation. For instance, in urinary tract infections (UTIs), where biofilm formation on indwelling catheters poses a significant risk, crystal violet staining of catheter samples helps clinicians identify and quantify biofilm-producing bacteria, guiding appropriate treatment strategies (Eberly et al., 2017). In addition to crystal violet, fluorescent stains such as SYTO 9 offer enhanced sensitivity and specificity for detecting metabolically active cells within biofilms (Stiefel, et al., 2015; Jin et al., 2005). SYTO 9 is a nucleic acid stain that penetrates bacterial cells, binding to DNA and emitting a green fluorescence when excited by light of an appropriate wavelength (McGoverin et al., 2020). Unlike crystal violet, which stains both living and dead cells indiscriminately, SYTO 9 selectively labels metabolically active cells within biofilms, providing valuable information on cell viability and activity (Stiefel, et al., 2015). An illustrative example of SYTO 9 staining is its application in environmental microbiology, particularly in assessing microbial communities in water treatment systems (Zand et al., 2021). By staining biofilm samples from water distribution pipes or filtration units with SYTO 9, researchers can visualize and quantify metabolically active bacteria, aiding in the optimization of treatment processes and ensuring water quality and safety (Zand et al., 2021). Moreover, the combination of crystal violet and SYTO 9 staining techniques offers a comprehensive approach to biofilm analysis, allowing researchers to assess both biomass and metabolic activity simultaneously and giving a better understanding of biofilm dynamics and function (Wu et al., 2019). For instance, in industrial settings such as food processing plants, where biofilm formation on surfaces can lead to contamination and product spoilage, dual staining with crystal violet and SYTO 9 enables the assessment of biofilm mass and viability. This information is invaluable for implementing effective cleaning and sanitation protocols to prevent biofilm-associated issues and ensure product quality and safety. Besides, novel staining approaches such as metabolic labelling and genetically encoded fluorescent proteins offer innovative solutions for biofilm detection and analysis. Metabolic labelling techniques involve incorporation of fluorescently labelled substrates or precursors into the biofilm matrix, allowing for real-time monitoring of biofilm growth and metabolic activity (Stiefel and Shen, 2022). For example, the use of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) as a metabolic stain enables visualization of respiring bacterial cells within the biofilm, providing insights into biofilm viability and activity (Schaule et al., 1993). On the other hand, genetically encoded fluorescent proteins such as green fluorescent protein (GFP) and red fluorescent protein (RFP) can be expressed by biofilm-forming bacteria to facilitate *in situ* visualization and tracking of biofilm formation and dynamics (Tomlin et al., 2004)). These genetically encoded reporters offer advantages such as high specificity, photostability, and compatibility with live-cell imaging techniques, making them powerful tools for studying biofilm behaviour in complex environments (Spacova et al., 2018; Shaner et al., 2008; Heydorn et al., 2000).

Staining dyes staining methods such as crystal violet and SYTO 9 serve as valuable tools for detecting bacterial biofilms, offering both advantages and limitations in their application. From traditional chromogenic stains to advanced fluorescence-based techniques, staining methods offer a diverse array of approaches for visualizing and analyzing biofilm architecture, composition, and dynamics. By combining staining methods with cutting-edge imaging technologies and innovative labelling strategies, scientists can gain deeper insights into the complex biology of bacterial biofilms and develop targeted interventions for controlling biofilm-related infections and environmental biofouling phenomena. One significant advantage of staining dyes is their versatility, as they can target specific components of

the biofilm matrix, such as bacterial cells, extracellular polymeric substances (EPS), or other biomolecules. This selective staining enables to visualize and differentiate between different biofilm constituents, providing insights into biofilm structure, composition, and spatial distribution. Additionally, staining dyes offer high sensitivity, allowing for the detection of even low levels of biofilm biomass or individual bacterial cells. The sensitivity is particularly advantageous in clinical and research settings, where accurate quantification of biofilm formation and growth is essential for understanding biofilm-related infections and developing effective treatment strategies.

However, the use of staining dyes for detecting bacterial biofilms also comes with certain limitations that need to be considered. One limitation is the potential for non-specific staining or background interference, which can arise from interactions between the dye and non-target components in the sample matrix (Haney, et al., 2018). Non-specific staining can lead to false-positive results and inaccurate interpretation of biofilm images, compromising the reliability of the staining technique (Silva et al., 2021). In addition, staining dyes may have variable affinities or specificities for different biofilm components, resulting in inconsistencies in staining intensity or contrast across samples (Stiefel and Shen, 2022). This variability can hinder quantitative analysis and comparison of biofilm samples, particularly in multi-species or complex microbial communities. Moreover, staining dyes may not provide information about the metabolic activity or physiological status of bacterial cells within the biofilm, as they primarily label structural components or biomolecules (Klinger-Strobel et al., 2016). This limitation necessitates complementary assays or techniques to assess biofilm viability and activity, such as metabolic stains or live/dead assays (Tawakoli et al., 2012). Some fluorescent dyes and proteins may interfere with cellular processes, possibly leading to toxicity or changes in cell behaviour, limiting characterization options (Wilson et al., 2017). Despite these limitations, staining dyes remain valuable tools for detecting bacterial biofilms, offering insights into biofilm structure, composition, and dynamics that are essential for understanding biofilm-associated infections and developing targeted interventions.

2.3.2. ATP-based assays

ATP-based chemical methods have emerged as powerful tools for detecting bacterial biofilms due to their sensitivity, specificity, and rapidity (Lee et al., 2010). These methods rely on the measurement of adenosine triphosphate (ATP), a universal energy molecule present in all living cells, including bacteria (Wilson et al., 2017). By quantifying ATP levels within biofilm samples, investigators can assess microbial viability and metabolic activity, providing valuable insights into biofilm formation, growth, and susceptibility to antimicrobial treatments (Zhang et al., 2023). One of the most commonly used ATP-based assays for biofilm detection is the bioluminescence assay, which utilizes luciferase enzymes to catalyze the conversion of ATP into light-emitting luciferin compounds (Wilson et al., 2017). This reaction produces a measurable bioluminescent signal proportional to the amount of ATP present in the sample, enabling rapid and quantitative assessment of biofilm viability. For example, the BacTiter-Glo™ Microbial Cell Viability Assay combines luciferase-based detection with a luminometer readout to quantify ATP levels in biofilm samples, allowing for high-throughput screening of antimicrobial agents and disinfectants (Bento et al., 2021; Sysel et al., 2021).

Another ATP-based approach for biofilm detection is the use of ATP bioluminescence imaging (ATP-BLI) techniques, which enable spatial mapping and visualization of microbial activity within biofilm structures (Doyle et al., 2004). ATP-BLI combines bioluminescence detection with advanced imaging technologies such as confocal laser scanning microscopy (CLSM) or bioluminescence microscopy to generate high-resolution images of ATP distribution within biofilm samples (Singh et al., 2024; Bueno, 2014; Yaseen et al., 2020; Yejiao et al., 2022). This allows researchers to assess biofilm heterogeneity, metabolic gradients, and spatial organization of bacterial communities, providing valuable insights into biofilm structure-function relationships. For instance, the COMSTAT (Computer-assisted Morphological Analysis of Biofilm Structure) software platform integrates ATP-BLI data with CLSM images to quantitatively analyze biofilm biomass, thickness, and roughness parameters, facilitating comprehensive characterization of biofilm architecture and dynamics (Heydorn et al., 2000). ATP-based methods can be adapted for *in situ* monitoring of biofilm formation and activity in real-time, enabling dynamic assessment of biofilm growth and response to environmental stimuli (Hong et al., 2021). These assays offer advantages over traditional culture-based methods for biofilm detection, as they provide rapid results without the need for lengthy incubation periods or specialized equipment (Chollet and Ribault, 2012). This makes ATP-based methods particularly suitable for high-throughput screening of biofilm-forming strains, as well as for on-site testing in clinical and environmental settings (Gatta et al., 2019). For example, the 3M™ Clean-Trace™ ATP Test System utilizes a handheld luminometer device to quantify ATP levels on surfaces, equipment, and environmental samples, providing rapid feedback on hygiene and cleanliness in healthcare facilities, food processing plants, and

other industrial settings (Boyce et al., 2010; Osimani et al., 2014; van Slooten et al., 2015; Ferreira et al., 2015).

ATP-based chemical methods stand out as rapid, sensitive, and straightforward approaches for detecting bacterial biofilms (Chollet and Ribault, 2012). Leveraging adenosine triphosphate (ATP) as a universal indicator of microbial metabolic activity, these methods provide swift insights into biofilm viability with minimal sample preparation. Their rapid turnaround time is particularly advantageous in clinical settings, where timely identification of biofilm-related infections is crucial for effective patient management. Furthermore, the high sensitivity of ATP-based assays enables the detection of even low levels of ATP present in small biofilm samples (Ihssen et al., 2021), facilitating the identification of viable bacteria within mature biofilms that may be challenging to detect using traditional culture-based methods. Additionally, the simplicity of ATP-based assays makes them accessible to a broad range of users, from healthcare professionals to researchers and industry personnel. With minimal sample preparation and instrumentation requirements, these methods can be easily adopted for routine monitoring of biofilm formation and activity in various settings. Their scalability also allows for high-throughput screening applications, making them suitable for large-scale studies evaluating the effectiveness of antimicrobial agents or disinfection protocols against biofilm formation (Gatta et al., 2019).

However, ATP-based chemical methods come with certain limitations that warrant consideration. One key limitation is their inability to differentiate between ATP derived from living bacterial cells and extracellular ATP released from lysed cells or residual ATP from non-viable cells (Arroyo et al., 2017). This limitation can lead to overestimation of biofilm viability, especially in samples with high background ATP levels (Wilson et al., 2017). Also, ATP-based assays may be susceptible to interference from environmental factors like pH, temperature, and chemical inhibitors, which can impact ATP extraction, detection, and quantification (Wilson et al., 2017). Besides, ATP-based methods may not offer insights into the spatial distribution or structural characteristics of biofilms, as they measure total ATP content rather than specific biofilm parameters. To address this limitation, combining ATP-based assays with complementary techniques such as microscopy or molecular imaging can provide a more comprehensive understanding of biofilm architecture and microbial distribution (Hasan et al., 2017). Lastly, the variable sensitivity of ATP-based assays to different bacterial species or strains necessitates validation against reference methods for accurate interpretation of results. Despite these limitations, ATP-based chemical methods remain valuable tools for detecting bacterial biofilms, offering rapid, sensitive, and quantitative assessment of biofilm viability across various applications.

2.4. Immunological methods

2.4.1. Enzyme-linked immunosorbent assay (ELISA) based techniques

Enzyme-Linked Immunosorbent Assay (ELISA) based techniques represent a powerful approach for detecting bacterial biofilms, offering high specificity, sensitivity, and versatility in their application. ELISA relies on the binding affinity between antibodies and specific antigens to detect and quantify target molecules within complex samples. In the context of bacterial biofilms, ELISA can be adapted to detect various biofilm components, including bacterial cells, extracellular polymeric substances (EPS), and specific biomolecules such as proteins, polysaccharides, or nucleic acids. One common ELISA-based approach for biofilm detection involves immobilizing antibodies against biofilm components onto a solid support, such as a microtiter plate or membrane, and then detecting the binding of target molecules using enzyme-conjugated secondary antibodies. The enzyme substrate produces a measurable signal, typically a colorimetric or fluorescent readout, proportional to the amount of target molecules present in the sample. In a study by Singh et al. (2021), DC-SIGN can recognize planktonic *Pseudomonas aeruginosa* cultures, unlike MR and Dectin-2, depending on the common polysaccharide antigen. In biofilms, DC-SIGN, MR, and Dectin-2 ligands show distinct clustering, with DC-SIGN also present among bacterial aggregates. All three receptors bind to carbohydrates from *P. aeruginosa* biofilms. Similarly, ELISA-based techniques have been used to detect and quantify bacterial biofilms in various other contexts, including medical device-associated infections, environmental biofouling, and food safety monitoring (Ma and Katzenmeyer-Pleuss, 2017; Canciu et al., 2021; Nivens et al., 2009). In another study by Wang et al. (2013), ELISA was employed to assess biofilm formation on patients having periprosthetic infections (PPI) commonly caused by *Staphylococcus epidermidis*. The researchers demonstrated that ELISA could effectively detect and quantify elevated levels of *S. epidermidis* anti-extracellular protein IgG in infected patients when compared to the control group, facilitating early diagnosis and treatment of biofilm-related complications.

Despite their numerous advantages, ELISA-based techniques for detecting bacterial biofilms also have several limitations that should be considered. One limitation is the potential for cross-reactivity or non-specific binding between antibodies and non-target molecules present in the sample matrix (Harro et al., 2020; Gu et al., 2005). This can lead to false-positive results and inaccurate interpretation of

biofilm data, particularly in complex or heterogeneous samples. Additionally, ELISA assays may require optimization and validation for each specific biofilm target and sample type to ensure reliable and reproducible results (Extremina et al., 2011). Moreover, ELISA-based techniques may have limited dynamic range and sensitivity for detecting low levels of biofilm components (Harro et al., 2020; Pu et al., 2020), particularly in samples with high background interference or low target abundance. This can pose challenges for accurate quantification of biofilm biomass or composition, especially in clinical or environmental samples with variable biofilm densities or compositions.

Finally, Enzyme-Linked Immunosorbent Assay (ELISA) based techniques offer a valuable tool for detecting bacterial biofilms, providing high specificity, sensitivity, and versatility in their application (Selan et al., 2008). By leveraging the binding affinity between antibodies and specific biofilm components, ELISA allows for the detection and quantification of target molecules within complex samples, facilitating insights into biofilm formation, composition, and dynamics (Estellés et al., 2016). ELISA remains a valuable and widely used method for studying bacterial biofilms in various research, clinical, and industrial settings, offering valuable insights into biofilm-associated infections, environmental biofouling, and food safety monitoring.

2.4.2. Immunofluorescence microscopy

Immunofluorescence microscopy-based techniques represent a powerful and versatile approach for detecting bacterial biofilms, offering high sensitivity, spatial resolution, and multiplexing capabilities. Immunofluorescence microscopy relies on the specific binding between fluorescently labeled antibodies and target antigens within the biofilm matrix, allowing for visualization and characterization of biofilm structure, composition, and dynamics (Shakes et al., 2012). One common immunofluorescence microscopy-based approach for biofilm detection involves labeling biofilm components with fluorophore-conjugated antibodies and then visualizing them using a fluorescence microscope. This technique enables researchers to localize and quantify specific biomolecules within the biofilm matrix with high specificity and spatial resolution (Ozer et al., 2021).

Seviour et al. (2021) used immunofluorescence microscopy to detect and visualize the distribution of extracellular DNA (eDNA) within *Pseudomonas aeruginosa* biofilms. The researchers labeled eDNA using a fluorophore-conjugated DNA-binding dye and then visualized its spatial distribution within the biofilm matrix using fluorescence microscopy. This approach revealed that eDNA was localized primarily to the periphery of the biofilm structure, suggesting its role in biofilm stability and integrity. Similarly, immunofluorescence microscopy-based techniques have been used to study biofilm formation and development in various other bacterial species and environmental conditions. For instance, in a study by Hu et al. (2013), immunofluorescence microscopy was employed to investigate the role of specific extracellular proteins in *Streptococcus mutans* biofilm formation on dental surfaces. The researchers labeled biofilm samples with fluorophore-conjugated antibodies targeting surface adhesins and exopolysaccharides produced by *S. mutans*, allowing for visualization and quantification of biofilm biomass and composition. By using fluorophore-conjugated antibodies with distinct emission spectra, investigators can label and distinguish different biomolecules within the biofilm matrix, such as bacterial cells, EPS, and specific proteins or nucleic acids. This multiplexing capability enables comprehensive characterization of biofilm architecture and composition, providing valuable insights into biofilm structure-function relationships and microbial interactions (Venkateshaiah et al., 2020).

Despite their numerous advantages, immunofluorescence microscopy-based techniques for detecting bacterial biofilms also have several limitations that should be considered. One limitation is the potential for nonspecific binding or background fluorescence, which can arise from autofluorescence of sample components or nonspecific interactions between fluorophores and non-target molecules. This can lead to false-positive signals and inaccurate interpretation of biofilm images, particularly in samples with high background fluorescence (Luan et al., 2016). Additionally, immunofluorescence microscopy may require careful optimization of labeling protocols and imaging parameters to achieve optimal signal-to-noise ratios and image quality (Harms, et al., 2023).

Moreover, immunofluorescence microscopy is limited by its two-dimensional imaging capabilities, which may not fully capture the three-dimensional architecture and spatial heterogeneity of complex biofilm structures. This limitation can be partially addressed by using confocal laser scanning microscopy (CLSM), which allows for optical sectioning and three-dimensional reconstruction of biofilm images (Franklin et al., 2015). However, CLSM techniques may be technically challenging and require specialized instrumentation and expertise (Relucenti et al., 2021).

Conclusively, immunofluorescence microscopy-based techniques offer a powerful and versatile approach for detecting bacterial biofilms, providing high sensitivity, spatial resolution, and multiplexing capabilities (Richter-Dahlfors et al., 2023). By leveraging the specific binding between fluorophore-conjugated antibodies and target antigens within the biofilm matrix, immunofluorescence microscopy

enables visualization and characterization of biofilm structure, composition, and dynamics (Relucenti et al., 2021). Despite their limitations, immunofluorescence microscopy-based techniques remain valuable tools for studying bacterial biofilms in various research, clinical, and industrial settings, offering insights into biofilm-associated infections, environmental biofouling, and biomedical applications (Johnson and Criss, 2013; Legner et al., 2020; Sugimoto et al., 2016).

2.5. Molecular methods

2.5.1. Polymerase chain reaction (PCR) based

Polymerase Chain Reaction (PCR)-based techniques have emerged as indispensable tools for detecting bacterial biofilms, offering unparalleled sensitivity, specificity, and versatility in their application (Millar et al., 2007). By amplifying specific DNA sequences within the biofilm matrix, PCR allows for the detection and quantification of target genes associated with biofilm formation, virulence, antibiotic resistance, and other phenotypic traits (Kırmusaoğlu, 2019). A study by DeLeo et al. (2023) exemplifies the utility of PCR in detecting biofilm-associated genes in clinical isolates of *Staphylococcus aureus* from patients with chronic wound infections. The researchers utilized multiplex PCR assays targeting genes encoding adhesion proteins, exopolysaccharides, and biofilm regulatory factors, demonstrating the presence of biofilm-related genetic markers in the majority of isolates (Demir et al., 2020; Mahmoudi et al., 2019; Budzyńska et al., 2021). Similarly, Le Gall et al. (2013) employed quantitative real-time PCR (qPCR) to quantify the expression of virulence genes in biofilms formed by clinical isolates of *Pseudomonas aeruginosa* from cystic fibrosis patients.

By designing qPCR assays targeting multiple biofilm-associated genes, including those involved in quorum sensing, exopolysaccharide synthesis, and antibiotic resistance, the researchers provided a comprehensive profile of biofilm phenotypes and their correlation with clinical outcomes. Furthermore, PCR-based techniques have been adapted for the rapid and sensitive detection of bacterial biofilms in environmental samples (Golpayegani et al., 2019). In a similar manner, Shi and Shi (2022) developed a lateral flow immunoassay for the visual and quantitative detection of viable *Listeria monocytogenes* cells and biofilms. It combines propidium monoazide-loop-mediated isothermal amplification (PMA-LAMP) with nanozyme technology. PMA-LAMP identified viable *L. monocytogenes* using FITC- and BIO-modified primers. Fe_3O_4 nanoparticles with optimized properties were combined with LAMP products and captured by an anti-FITC antibody on a nanozyme strip, offering stability, specificity, and visualization for *L. monocytogenes* quantification (detection limit: 10 CFU mL^{-1}). The strip effectively detected *L. monocytogenes* biofilms on stainless steel and lettuce surfaces. Additionally, advances in PCR technology have led to the development of novel techniques such as digital droplet PCR (ddPCR), offering absolute quantification of target DNA molecules without the need for standard curves or reference samples. Zheng et al. (2021) demonstrated this technology by employing ddPCR to quantify antibiotic resistance genes in biofilms formed by multidrug-resistant strains of *Acinetobacter baumannii* on medical device surfaces. Their findings underscored the ability of ddPCR to accurately detect and quantify target genes, providing insights into the prevalence and persistence of antibiotic resistance in biofilm communities. Moreover, recent advancements in PCR-based techniques have focused on enhancing their sensitivity and specificity for detecting low-abundance biofilm components or rare microbial species. Tomás et al. (2018) investigated the bacterial communities in biofilms formed on external substrates (substrate-formed biofilms) and teeth (supragingival tooth-formed biofilms) in the same group of individuals. Nested PCR was employed to compare both the viability and diversity of the biofilm communities.

The researchers designed nested primer sets targeting conserved regions of bacterial 16S rRNA genes, allowing for selective amplification of target sequences from complex microbial communities. This approach enabled sensitive detection of low-abundance bacterial species within the biofilm matrix, providing insights into microbial composition and diversity. Furthermore, PCR-based techniques have been integrated with other molecular biology methods, such as next-generation sequencing (NGS) and metagenomic analysis, to provide comprehensive insights into biofilm structure, composition, and dynamics. For instance, in a study by Nayak et al. (2023), PCR amplification of 16S rRNA genes followed by NGS was used to characterize the microbial communities associated with chronic wound biofilms. The researchers identified diverse bacterial taxa within the biofilm matrix, including both known pathogens and previously unrecognized species, highlighting the complexity of biofilm-associated infections. In a different context, Shemesh et al. (2007) applied PCR-based techniques to investigate biofilm formation and antibiotic resistance in *Streptococcus mutans* on dental surfaces. Utilizing conventional PCR and qPCR assays targeting genes associated with biofilm adhesion and extracellular polysaccharide production, the researchers elucidated the molecular mechanisms underlying biofilm development and its impact on dental caries. Similarly, Terefework et al. (2008) utilized multiplex ligation-dependent probe amplification (MLPA) to quantify 9 bacterial species in oral biofilms based on their 16S rDNA sequences.

MLPA detected 10 pg DNA with clear signals and showed cost-effectiveness versus qPCR. It revealed signal variations in biofilm DNA and saliva DNA from different donors, demonstrating its utility in quantifying microbial shifts. Moreover, the versatility of PCR-based techniques extends to the detection and quantification of specific bacterial species within biofilm communities. Tak et al. (2023) conducted a study wherein droplet digital PCR (ddPCR) was employed to detect and quantify the presence of specific bacterial pathogens in biofilms formed by periprosthetic joint infection (PJI). ddPCR detected 400 attograms of target DNA, surpassing real-time PCR with synthesized plasmid by over 10 times. It identified target regions from genomic DNA as low as 50 femtograms for *E. coli*, 70 femtograms for *S. epidermidis*, and 90 femtograms for *S. aureus*, showing its potential for early PJI detection.

Despite their numerous advantages, PCR-based techniques for detecting bacterial biofilms also have several limitations that should be considered. One limitation is the potential for false-positive or false-negative results due to sample contamination, primer design errors, or PCR inhibitors present in the sample matrix (Kibbee and Örmeci, 2017; Hall-Stoodley et al., 2012). Careful experimental design and validation are essential to ensure the reliability and reproducibility of PCR-based assays. Additionally, PCR-based techniques may be limited by their dependence on target gene sequences, which may vary between bacterial strains or biofilm phenotypes, leading to challenges in the universal detection of biofilm-associated genes across different species or environmental conditions (Abdelraheem et al., 2020). Moreover, PCR-based assays may require specialized equipment, reagents, and expertise, which may limit their accessibility and scalability in resource-limited settings (Chakraborty, 2024). Furthermore, PCR-based techniques may not provide information about the spatial distribution or structural characteristics of biofilms, as they primarily detect and quantify genetic markers rather than visualizing biofilm architecture or microbial interactions (Sanz and Köchling, 2007).

2.5.2. Fluorescence in situ hybridization (FISH)

Bacterial biofilms, having communities of microorganisms encased in a self-produced extracellular matrix, pose a significant challenge in diverse fields, ranging from healthcare and industry to environmental ecosystems. Their inherent resistance to traditional therapeutic strategies underscores the need for robust and reliable methods for their detection, identification, and characterization (Strelkova et al., 2013). Fluorescence in situ hybridization (FISH), a powerful molecular tool, emerges as a valuable weapon in this arsenal, offering unique advantages compared to conventional methods. One of the key strengths of FISH lies in its ability to directly visualize the target microbial populations *in situ* within the biofilm matrix. Unlike culture-based methods, which often underestimate bacterial diversity and viability due to selective growth requirements, FISH allows for the identification of specific bacterial populations regardless of their cultivability (Frickmann et al., 2017). This is achieved by employing fluorescently labeled probes, short, 18-25 bp sized, single-stranded DNA sequences complementary to the target ribosomal RNA (rRNA) – a highly conserved molecule present in all bacterial cells (Frickmann et al., 2017). By hybridizing with their specific targets within the biofilm, these probes enable researchers to visually distinguish between different bacterial species or functional groups through fluorescence microscopy (Frickmann et al., 2017). Recent advancements have further refined the efficacy of FISH in biofilm research.

Multiplex FISH (mFISH) allows for the simultaneous visualization of multiple bacterial populations within a single sample. This technique utilizes a combination of probes, each labeled with a distinct fluorophore, enabling researchers to differentiate between diverse bacterial community's residents within the biofilm (Thurnheer et al., 2004). Studies by Al-Ahmad et al. (2007) effectively employed mFISH to unravel the intricate microbial tapestry of *Streptococcus* spp., *Actinomyces naeslundii*, *Fusobacterium nucleatum* and *Veillonella* spp. in dental plaque biofilm, highlighting its potential for elucidating the complex interplay between various bacterial species within these biofilms, ultimately informing novel strategies for preventing implant-associated infections.

Furthermore, quantitative FISH (qFISH) has emerged as a powerful tool for quantifying specific bacterial populations within biofilms. By employing image analysis software, researchers can estimate the abundance of various bacterial species based on the intensity and number of fluorescent signals emitted by the probes bound to their targets (Kolpen et al., 2022). This approach has been utilized to assess the organization of bacteria in sputum samples from individuals with acute and chronic lung infections. Biofilms dominate both acute and chronic lung infections, with faster-growing bacteria enriched in biofilms similar to those in chronic infections. Lung inflammation is similar, but systemic markers are elevated only in acute infections. Beyond species identification and quantification, FISH can also be used to investigate the spatial distribution of bacterial populations within biofilms (Barbosa et al., 2023). By analyzing the 3-D structure of the biofilm using techniques like confocal laser scanning microscopy (CLSM) in conjunction with FISH, researchers can gain valuable insights into the spatial organization of

different bacterial communities within the biofilm matrix (Barbosa et al., 2023). Studies by Karygianni et al. (2014) employed CLSM-mFISH to early (*Streptococcus* spp., *Actinomyces naeslundii*) and late colonizers (*Fusobacterium nucleatum*, *Veillonella* spp.) of in situ-formed oral biofilms, highlighting the spatial distribution of highly variable phenotypes found in multispecies oral biofilms. The versatility of FISH extends beyond bacterial identification. By employing probes targeting specific genes or functional markers, researchers can gain insights into the physiological state of bacterial populations within biofilms. Probes targeting genes associated with antibiotic resistance or virulence factors can be utilized to identify potentially pathogenic subpopulations within biofilms. This approach was employed by Yang et al. (2008) to identify antibiotic-resistant bacterial subpopulations within biofilms associated with cystic fibrosis, paving the way for the development of personalized therapeutic strategies that take into account the specific resistance profiles of these subpopulations, thereby improving treatment efficacy and patient outcomes.

However, it is crucial to acknowledge the limitations associated with FISH techniques. The specificity of the probes remains paramount, as any non-specific binding can lead to misinterpretation of data and inaccurate conclusions (Bishop, 2010). Careful design and rigorous validation of probes are essential to ensure accurate identification and prevent misleading results (Legendre et al., 2013). Additionally, the processes of fixation and permeabilization, necessary for probe penetration into the biofilm matrix, can potentially alter the structural integrity of biofilms, potentially affecting the accessibility of target molecules for probe binding and leading to underestimation of specific bacterial populations (Barbosa et al., 2023). The importance of optimizing fixation and permeabilization protocols for different biofilm types and target organisms to minimize such artifacts and ensure reliable data acquisition, thereby enhancing the accuracy and reproducibility of the technique (Rocha et al., 2018). Furthermore, FISH can be labour-intensive and requires specialized equipment and expertise. Additionally, the interpretation of complex mFISH data can be challenging and may necessitate advanced image analysis skills and training (Kwasny et al., 2012).

2.6. Emerging technologies

2.6.1. Biosensor

Biosensor-based techniques have emerged as powerful tools for the detection and characterization of bacterial biofilms, offering rapid, sensitive, and specific methods for identifying and quantifying these complex microbial communities (Pu et al., 2021). By exploiting the biological interactions between target molecules and biorecognition elements, biosensors enable real-time monitoring of biofilm formation, providing valuable insights into their structure, composition, and dynamics (Funari and Shen, 2022). Various biosensor platforms, including optical, electrochemical, and piezoelectric sensors, have been developed and tailored for biofilm detection, each offering unique advantages in terms of sensitivity, specificity, and portability (Banakar et al., 2022).

Optical biosensors, such as surface plasmon resonance (SPR) and fluorescence-based systems, utilize light-matter interactions to detect biomolecular binding events within biofilms. SPR biosensors, for instance, rely on changes in refractive index upon biomolecule binding to a sensor surface, enabling label-free detection of biofilm components in real time (Babicheva, 2023). Fluorescence-based biosensors, on the other hand, utilize fluorescent probes or labels that emit light upon binding to target molecules, allowing for sensitive and multiplexed detection of specific biofilm constituents (Péter et al., 2022). These optical techniques offer high sensitivity and specificity, making them valuable tools for studying biofilm formation and dynamics in various environments, from medical to environmental and industrial settings (Funari and Shen, 2022). Electrochemical biosensors exploit changes in electrical properties, such as current or voltage, upon biomolecular interactions at electrode surfaces (Huang et al., 2015). This includes techniques like amperometry, voltammetry, and impedimetry, which can detect biofilm-related molecules such as enzymes, metabolites, or DNA/RNA fragments with high sensitivity and selectivity (Parlak and Richter-Dahlfors, 2020; McGlennen, 2023). Electrochemical biosensors offer advantages such as rapid response, low cost, and ease of miniaturization, making them suitable for on-site monitoring of biofilm-related processes in real-world applications (Menon et al., 2020; Ghorbani-Bidkorbeh, 2015). Piezoelectric biosensors, based on the principle of mass-induced frequency changes in piezoelectric crystals upon biomolecular binding, provide another powerful approach for biofilm detection (Chen et al., 2023). Quartz crystal microbalance (QCM) and surface acoustic wave (SAW) sensors are commonly used in biofilm research, offering high sensitivity and real-time monitoring capabilities. These sensors can detect minute changes in biofilm mass, thickness, or viscoelastic properties, providing valuable information on biofilm formation kinetics and adhesion forces (Cooper and Singleton, 2007; Rocha-Gaso et al., 2009).

Despite their numerous advantages, biosensor-based techniques for detecting bacterial biofilms also face certain limitations. One major challenge is the complexity and heterogeneity of biofilm matrices, which can affect the accessibility and binding kinetics of target molecules to bio-recognition

elements, leading to false-positive or false-negative results (Paniel et al., 2013). Additionally, biosensors may require optimization and validation for specific biofilm samples or environmental conditions, limiting their generalizability and scalability for routine use. Furthermore, the sensitivity and selectivity of biosensors may be influenced by interfering substances or background noise present in complex sample matrices, necessitating robust signal processing and data analysis techniques (Pereira and Melo, 2023; Prabowo et al., 2021).

In conclusion, biosensor-based techniques offer powerful tools for detecting and studying bacterial biofilms, providing rapid, sensitive, and specific methods for real-time monitoring of biofilm formation and dynamics (Pu et al., 2021). Optical, electrochemical, and piezoelectric biosensors have been developed and tailored for biofilm detection, each offering unique advantages in terms of sensitivity, specificity, and portability (Banakar et al., 2022). Despite their limitations, biosensors hold great promise for advancing our understanding of biofilm-related processes and facilitating the development of novel strategies for biofilm control and management in various applications (Ivanova et al., 2017).

2.6.2. Acoustic techniques

Acoustic techniques have emerged as promising tools for the detection and characterization of bacterial biofilms, offering non-invasive, real-time, and label-free methods for assessing biofilm formation and properties (Subramanian et al., 2020). These techniques exploit the interactions between sound waves and biofilm structures (Lui et al., 2023), leveraging principles of acoustics to probe biofilm-related processes at the microscale. Various acoustic methods, including ultrasound, acoustic microscopy, and acoustic sensors, have been developed and utilized for biofilm detection and monitoring, each offering unique advantages and capabilities (Lui et al., 2023; Anastasiadis et al., 2014; George et al., 2006). Ultrasound-based techniques utilize high-frequency sound waves (>20 kHz) to penetrate biofilm matrices and generate images based on the reflection and scattering of acoustic signals (Au and Zwank, 2020). Ultrasound imaging can provide information on biofilm thickness, density, and structure, allowing for non-destructive visualization and characterization of biofilm architecture (Iqbal et al., 2013). By measuring ultrasound wave propagation parameters such as attenuation, velocity, and backscatter, researchers can assess biofilm properties such as porosity, viscosity, and mechanical strength, providing insights into biofilm development and stability over time (Pereira and Melo, 2009). Acoustic microscopy extends the capabilities of ultrasound imaging by offering higher spatial resolution and sensitivity to structural details within biofilm samples. By employing focused ultrasound beams and sophisticated imaging algorithms, acoustic microscopes can generate high-resolution images of biofilm morphology, revealing fine-scale features such as cell clusters, extracellular matrix components, and microcolonies (Anastasiadis et al., 2014). Acoustic microscopy techniques such as scanning acoustic microscopy (SAM) and photoacoustic microscopy (PAM) enable researchers to visualize biofilm dynamics in real time and assess the effects of environmental factors or antimicrobial treatments on biofilm structure and integrity (Yu, 2020; Yao and Wang, 2013). Acoustic sensors represent another class of acoustic techniques for biofilm detection, relying on changes in acoustic properties such as frequency, amplitude, or phase in response to biofilm-related events (Kim et al., 2016; Hazan et al., 2006). These sensors can detect biofilm formation, growth, and detachment by monitoring changes in acoustic signals caused by biomass accumulation or structural alterations within biofilm matrices. Surface acoustic wave (SAW) sensors, quartz crystal microbalance (QCM) sensors, and resonant microcantilever sensors are examples of acoustic sensor platforms used for biofilm detection and monitoring in various applications, including medical diagnostics, environmental monitoring, and industrial process control.

Despite their numerous advantages, acoustic techniques for detecting bacterial biofilms also face certain limitations. One major challenge is the complexity and heterogeneity of biofilm structures, which can affect the propagation and interpretation of acoustic signals, leading to uncertainties or inaccuracies in biofilm detection and characterization (Achinas et al., 2020). Additionally, acoustic techniques may require specialized equipment, expertise, and calibration procedures for optimal performance, limiting their accessibility and scalability for routine biofilm analysis. Furthermore, acoustic signals may be influenced by environmental factors such as temperature, humidity, and background noise, necessitating careful experimental design and control measures to ensure reliable and reproducible results (Funari and Shen, 2022).

In conclusion, acoustic techniques offer valuable approaches for detecting and studying bacterial biofilms. Ultrasound imaging, acoustic microscopy, and acoustic sensors enable researchers to visualize biofilm morphology, monitor biofilm dynamics, and quantify biofilm-related parameters with high sensitivity and spatial resolution (Cruz et al., 2021). Despite their limitations, acoustic techniques hold great promise for advancing our understanding of biofilm-related processes and facilitating the development of novel strategies for biofilm control and management in various applications.

2.6.3. Mass spectrometry

Mass spectrometric techniques have emerged as powerful tools for the detection and characterization of bacterial biofilms, offering high sensitivity, specificity, and versatility for analyzing biofilm composition and dynamics (Sportelli et al., 2022). These techniques rely on the principles of mass spectrometry to identify and quantify biomolecules within biofilm matrices, providing valuable insights into the microbial communities, metabolic pathways, and molecular interactions underlying biofilm formation and behaviour (Dunham et al., 2017). Various mass spectrometric methods, including matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), liquid chromatography-mass spectrometry (LC-MS), and gas chromatography-mass spectrometry (GC-MS), have been developed and utilized for biofilm analysis, each offering unique advantages and capabilities.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a powerful technique for rapid and label-free analysis of biofilm samples, allowing for the direct detection and identification of biomolecules such as proteins, lipids, and nucleic acids (Masyuko et al., 2014). By coupling MALDI-MS with high-resolution mass analyzers and bioinformatics tools, researchers can profile the molecular composition of biofilms, identify specific biomarkers, and elucidate biochemical pathways associated with biofilm development and resistance (Janiszewska et al., 2022). MALDI-MS imaging further extends the capabilities of MALDI-MS by enabling spatial mapping of biomolecules within biofilm samples, providing insights into their distribution and localization at the microscale level.

Liquid chromatography-mass spectrometry (LC-MS) combines the separation power of liquid chromatography with the detection sensitivity and specificity of mass spectrometry, offering a comprehensive approach for analyzing complex biofilm samples (Favre et al., 2017). LC-MS allows for the identification and quantification of a wide range of biomolecules, including proteins, peptides, metabolites, and lipids, in biofilm extracts or culture supernatants (Nakayasu et al., 2016). By employing multidimensional chromatography and tandem mass spectrometry (MS/MS) techniques, researchers can achieve deep coverage of the biofilm proteome and metabolome, uncovering novel biomarkers and metabolic pathways associated with biofilm development and virulence (Ribeiro et al., 2019).

Gas chromatography-mass spectrometry (GC-MS) is another widely used technique for analyzing volatile and semi-volatile compounds in biofilm samples, such as volatile organic compounds (VOCs) and fatty acids (Lahiri et al., 2021). GC-MS enables the identification and quantification of these compounds with high sensitivity and selectivity, providing insights into microbial metabolism, biofilm activity, and environmental influences (Thorn and Greenman, 2012). By analyzing the volatile profile of biofilms, researchers can discriminate between different bacterial species or strains, monitor biofilm growth and metabolism in real time, and assess the effects of antimicrobial agents or environmental stressors on biofilm viability and physiology (Funari and Shen, 2022; Zihan et al., 2023).

Despite their numerous advantages, mass spectrometric techniques for detecting bacterial biofilms also face certain limitations. One of the major challenge is the intricacy and heterogeneity of biofilm matrices, which can affect the extraction, ionization, and detection of biomolecules by mass spectrometry, leading to variability or inconsistencies in analytical results (Magana et al., 2018). Additionally, mass spectrometric methods may require extensive sample preparation, instrument optimization, and data processing procedures, which can be time-consuming and resource-intensive, particularly for high-throughput analysis of large sample cohorts (Sakallioglu et al., 2022). Furthermore, the sensitivity and specificity of mass spectrometric techniques may be influenced by matrix effects, ion suppression, or interference from background contaminants, necessitating careful validation and quality control measures to ensure accurate and reliable results (Lehotay et al., 2015).

In conclusion, mass spectrometric techniques offer powerful and versatile approaches for detecting and characterizing bacterial biofilms, providing high sensitivity, specificity, and molecular resolution for analyzing biofilm composition and dynamics (Sportelli et al., 2022). MALDI-MS, LC-MS, and GC-MS enable researchers to identify biomarkers, elucidate biochemical pathways, and monitor microbial metabolism within biofilms, offering valuable insights into biofilm-related processes and facilitating the development of novel strategies for biofilm control and management in various applications. Despite their limitations, mass spectrometric techniques hold great promise for advancing our understanding of biofilm biology and facilitating translational research efforts aimed at combating biofilm-associated infections and environmental contamination.

3. Soil and Microbes

Thousands of potentially interacting species make up microbial communities, which perform vital ecosystem functions. Studying the functional characteristics of microorganisms may provide insight into the creation and upkeep of these complex ecosystems (Treseder and Lennon, 2015). The distribution of features could be a reflection of trade-offs and adaptations that affect ecological and evolutionary processes crucial for the formation of communities along environmental gradients (Lennon and Denef, 2015). At last, trait-based methods offer a structure for forecasting the manner and timing in which microbial taxa ought to impact ecosystem

performance (Krause et al., 2014). Biofilm formation is a property that may have significant effects on processes occurring at the population and community levels because it alters microbial interactions. For instance, the close proximity of individuals within a biofilm might enable communication and syntrophic interactions among microbes. One characteristic that seems to be especially prevalent in soil-dwelling bacteria is the development of biofilms. Although their chemical composition and structure may vary, biofilms often possess hydrophobic characteristics that aid in the retention of water within the soil matrix (Chang et al., 2007; Kun et al., 2023). This is significant because low moisture conditions frequently hinder the motility and limit substrate diffusion of soil microorganisms (Potts, 1994). Furthermore, the development of biofilms is advantageous as it mitigates desiccation stress in soil conditions. For instance, in a phylogenetically varied group of soil microbes, biofilm development was associated with the moisture niche (Lennon et al., 2012). More specifically, bacteria that produced more biofilms exhibited a drier optimum and were able to withstand a wider range of soil moisture. On the other hand, little is known about species interactions and trade-offs related to the formation of bacterial biofilms in soils.

4. Considerations and future directions

Detecting bacterial biofilms poses significant challenges due to their complex and dynamic nature (Abdelhamid and Yousef, 2023). However, advancements in detection strategies offer promising avenues for overcoming these challenges and improving our ability to identify and characterize biofilms in various environments (Cavalheiro and Teixeira, 2018). Several key considerations and future directions in biofilm detection have emerged, including the adoption of multimodal approaches, development of *in situ* detection methods, targeting species-specific biofilm components, and the automation and miniaturization of detection platforms. Each of these areas holds potential for enhancing our understanding of biofilm biology and guiding the development of targeted strategies for biofilm control and management.

4.1. Multimodal approaches

One of the key considerations in biofilm detection is the adoption of multimodal approaches that combine different detection strategies to provide complementary information and overcome individual limitations. For example, integrating optical, electrochemical, and acoustic techniques can offer synergistic advantages in terms of sensitivity, specificity, and spatial resolution (Juárez, 2023; Ortkrass et al., 2024; Kreger et al., 2020). By combining the strengths of different detection modalities, researchers can enhance the accuracy and reliability of biofilm detection, allowing for comprehensive characterization of biofilm structure, composition, and behaviour. Multimodal approaches also enable researchers to leverage the unique capabilities of each technique to address specific challenges associated with biofilm detection in different environments, such as medical devices or natural settings (Tran and Prindle, 2021).

4.2. In situ detection

Another important consideration in biofilm detection is the development of methods for in situ detection in complex environments such as medical devices or natural settings (Xu et al., 2020). Traditional laboratory-based detection methods often require the removal of biofilm samples from their native environment, which can alter their structure and composition and limit the relevance of the obtained results. In situ detection methods, on the other hand, enable real-time monitoring of biofilm formation and dynamics within their natural context, providing valuable insights into biofilm behaviour and response to environmental stimuli (Azeredo et al., 2017). Techniques such as fluorescence in situ hybridization (FISH), confocal laser scanning microscopy (CLSM), and surface-enhanced Raman spectroscopy (SERS) have been developed for in situ detection of biofilms in various environments, offering non-invasive and high-resolution imaging capabilities (Carrascosa et al., 2021).

4.3. Species-specific detection

Targeting specific biofilm components or activities unique to specific pathogens is another important consideration in biofilm detection. Traditional detection methods often lack specificity and can fail to distinguish between different microbial species or strains within a biofilm community (Magana et al., 2018). By developing species-specific detection strategies that target biomolecules or metabolic pathways unique to specific pathogens, researchers can improve the specificity and accuracy of biofilm detection, enabling more targeted treatment strategies (Koo et al., 2017) such as targeting species-specific biomarkers or virulence factors can facilitate the early detection of pathogenic biofilms and guide the selection of appropriate antimicrobial agents or therapeutic interventions (Antypas et al., 2018).

4.4. Automation and miniaturization

Automation and miniaturization of detection platforms represent another promising direction for biofilm detection, offering potential benefits in terms of rapid, point-of-care diagnosis and monitoring (Vasala et al., 2020). Traditional detection methods often require specialized equipment, trained personnel, and lengthy processing times, limiting their suitability for on-site or field-based

applications. Automated and miniaturized detection platforms, on the other hand, offer advantages such as simplicity, portability, and scalability, making them well-suited for decentralized and resource-limited settings (Haney et al., 2017). By integrating sample preparation, detection, and data analysis into a single automated platform, researchers can streamline the detection process and facilitate rapid decision-making in clinical or environmental settings (Kaushik et al., 2018). Moreover, miniaturized detection platforms enable the development of point-of-care devices that can be deployed in remote or resource-constrained areas, providing timely and accurate diagnosis of biofilm-related infections or environmental contamination (Ardila et al., 2023).

5. Conclusion

In conclusion, the detection of bacterial biofilms presents a multifaceted challenge that requires a diverse array of strategies to unveil the hidden world of microbial aggregates. Complex populations of microorganisms covered in a matrix of self-produced extracellular polymeric substance (EPS) are known as bacterial biofilms. Traditional culture-based methods, while cost-effective and familiar, suffer from limitations such as underestimation of biomass and limited species composition information. Physical methods offer rapid visual inspection and high-resolution imaging capabilities, yet they may lack sensitivity or require specialized equipment. Chemical methods, including staining techniques and ATP-based assays, provide simple and quantitative insights into biofilm biomass and viability but face challenges in differentiating live and dead cells. Immunological methods, such as ELISA and immunofluorescence microscopy, offer high sensitivity and specificity but can be expensive and require expertise. Molecular methods like PCR, qPCR, and FISH enable specific detection and identification of biofilm-forming bacteria, although they may require specialized primers, instrumentation, or optimization. Emerging technologies, including biosensors, acoustic techniques, and mass spectrometry, hold promise for rapid, real-time monitoring and comprehensive molecular analysis of biofilms but require further development and validation. Moving forward, considerations such as multimodal approaches, in situ detection methods, species-specific targeting, and automation and miniaturization will be critical for advancing biofilm detection capabilities. By embracing these future directions, researchers can enhance our understanding of biofilm biology and develop targeted strategies for combating biofilm-related infections and environmental contamination.

Declarations

Ethics approval and consent to participate

Consent for publication: The article contains no such material that may be unlawful, defamatory, or which would, if published, in any way whatsoever, violate the terms and conditions as laid down in the agreement.

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