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Methodological Approaches for Investigating the Effects of Bioactive Compounds on Quorum Sensing-Related Virulence Factors in Pathogenic Bacteria

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Key words

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Abstract

The global rise of antimicrobial resistance has intensified the search for therapeutic strategies that reduce bacterial pathogenicity without relying solely on bacterial killing. Quorum sensing (QS), a cell-density-dependent communication system, regulates key virulence traits including biofilm formation, motility, toxin production, extracellular enzyme secretion, immune evasion, metabolic adaptation, and antimicrobial tolerance. As a result, QS has become a promising target for anti-virulence therapy. Numerous bioactive compounds, including phytochemicals, essential oils, microbial metabolites, antimicrobial peptides, quorum-quenching enzymes, synthetic inhibitors, and nanomaterials, have been investigated for their ability to interfere with QS-regulated pathways. However, validating anti-QS activity remains methodologically challenging because many putative QS inhibitors also affect bacterial growth, metabolism, membrane integrity, or biofilm stability, making it difficult to distinguish true communication interference from nonspecific antimicrobial effects. This review critically evaluates the methodologies used to investigate QS-related virulence inhibition in pathogenic bacteria. It examines key experimental parameters, including strain selection, inoculum standardization, MIC and sub-MIC determination, phenotypic and biosensor-based assays, signal molecule detection, molecular and omics approaches, advanced imaging, in vivo models, and computational tools. Particular emphasis is placed on the limitations of single-endpoint assays and the importance of integrating phenotypic, molecular, and functional evidence before classifying a compound as a genuine QS inhibitor. By identifying common sources of bias, reproducibility issues, and translational challenges, this review proposes a rigorous framework for the evaluation of anti-QS compounds and their potential application against clinically relevant bacterial pathogens.

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1. Introduction

Antimicrobial resistance (AMR) has emerged as one of the greatest threats to global public health, compromising the effectiveness of conventional antibiotics and increasing morbidity, mortality, and healthcare-associated costs worldwide (Murray et al., 2022; Naghavi et al., 2024). The rapid dissemination of multidrug-resistant (MDR) bacterial pathogens, combined with the declining discovery rate of novel antibiotics, has intensified the search for alternative therapeutic strategies

capable of controlling bacterial infections without exerting strong selective pressure for resistance development (Dehbanipour & Ghalavand, 2022). Among these alternative approaches, anti-virulence therapies targeting bacterial communication systems have attracted considerable scientific attention (Hetta et al., 2024).

Quorum sensing (QS) is a cell-density-dependent communication mechanism that enables bacteria to coordinate collective behaviors through the production, release, accumulation, and detection of signaling molecules known as autoinducers (Chu et al., 2024). Through QS systems, bacterial populations regulate the expression of a wide variety of virulence-associated traits, including biofilm formation, motility, toxin production, secretion systems, pigment synthesis, extracellular enzyme release, adhesion, immune evasion, and host colonization (Warrier et al., 2021). In many clinically important pathogens, QS contributes directly to pathogenicity, persistence, and antimicrobial tolerance, particularly in chronic and biofilm-associated infections (Sharma et al., 2023).

In Gram-negative bacteria, quorum sensing commonly relies on signaling molecules such as acyl-homoserine lactones (AHLs), autoinducer-2 (AI-2), and quinolone-based signals, whereas Gram-positive bacteria predominantly use autoinducing peptides (AIPs) and two-component regulatory systems (Chu et al., 2024). Complex and interconnected QS regulatory networks have been extensively described in major pathogens such as Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumannii, Klebsiella pneumoniae, and Escherichia coli (Zhang et al., 2025). These signaling pathways not only regulate bacterial virulence but also influence interspecies communication, microbial competition, microbiota interactions, and host-pathogen relationships (Cui et al., 2024).

Unlike conventional antibiotics that directly inhibit bacterial growth or viability, anti-QS strategies aim to attenuate pathogenicity by disrupting bacterial communication and virulence regulation without necessarily killing bacterial cells (Dehbanipour & Ghalavand, 2022). This antivirulence approach is considered particularly attractive because it may reduce the selective pressure associated with the emergence of antibiotic resistance while preserving beneficial microbiota (Hetta et al., 2024). Consequently, quorum sensing inhibition has become an increasingly important field in antimicrobial research, especially in the context of chronic infections, implant-associated infections, and biofilm-mediated diseases (Naga et al., 2024).

Over the past decade, a broad diversity of bioactive compounds has been investigated for their ability to interfere with QS systems and QS-regulated virulence factors. These compounds include plant-derived metabolites such as flavonoids, alkaloids, terpenoids, tannins, and essential oils; microbial metabolites including probiotics, postbiotics, bacteriocins, and biosurfactants; synthetic quorum sensing inhibitors; antimicrobial peptides; and nanostructured materials such as silver nanoparticles, zinc oxide nanoparticles, chitosan nanoparticles, and lipid-based nanocarriers (Papaneophytou, 2026; Hu et al., 2024). Numerous studies have demonstrated that these compounds can reduce biofilm formation, inhibit motility, suppress toxin production, interfere with signaling pathways, and modulate virulence gene expression in pathogenic bacteria (Gad et al., 2024; Talla et al., 2023).

Despite the growing number of anti-QS investigations, major methodological inconsistencies remain across the literature. Variations in bacterial strains, culture conditions, inoculum preparation, determination of sub-inhibitory concentrations, virulence assays, biosensor systems, molecular techniques, and data interpretation frequently limit reproducibility and complicate comparisons between studies (Naga et al., 2024). In many cases, reductions in virulence-associated phenotypes may result from indirect antibacterial effects rather than true quorum sensing inhibition (Hetta et al., 2024). Moreover, the lack of standardized protocols and harmonized experimental workflows represents a major challenge for the translation of anti-QS compounds from laboratory research to clinical applications (Dehbanipour & Ghalavand, 2022).

The increasing complexity of modern anti-virulence research has also expanded the range of analytical and methodological approaches used in this field. Contemporary studies increasingly integrate phenotypic assays, transcriptomics, proteomics, metabolomics, advanced microscopy, biosensor systems, molecular docking, artificial intelligence-assisted screening, and in vivo infection models to evaluate the anti-QS potential of bioactive compounds (Hu et al., 2024). Such multidisciplinary approaches provide deeper mechanistic insights into quorum sensing inhibition while improving the reliability and translational relevance of experimental findings.

Given the rapid expansion of anti-QS research and the methodological diversity observed in this field, there is a growing need for a comprehensive and critical review specifically focused on the experimental methodologies used to investigate the effects of bioactive compounds on quorum sensing-related virulence factors in pathogenic bacteria. While previous reviews have primarily focused on the biological activities or therapeutic potential of quorum sensing inhibitors, relatively few studies have systematically examined the methodological frameworks underlying anti-QS investigations (Naga et al., 2024).

Therefore, the present review aims to provide a comprehensive overview of the major methodological approaches employed in the evaluation of anti-quorum sensing and anti-virulence activities of bioactive compounds. Particular attention is given to experimental design, phenotypic assays, molecular and omics techniques, biosensor systems, advanced imaging technologies, *in vivo* models, and computational approaches used in contemporary anti-QS research. In addition, this review critically discusses the advantages, limitations, reproducibility challenges, and standardization needs associated with current methodologies while highlighting emerging technologies and future perspectives in the field of anti-virulence therapeutics.

To provide a comprehensive overview of the scientific rationale underlying anti-quorum sensing and anti-virulence research, Figure 1 summarizes the relationship between antimicrobial resistance, the limitations of conventional antibiotic therapies, and the emergence of quorum sensing-targeted interventions. The figure highlights the central role of QS in regulating bacterial pathogenicity, illustrates the principal mechanisms through which anti-QS compounds interfere with bacterial communication and virulence expression, and presents the diverse classes of bioactive agents currently investigated as quorum quenchers. Furthermore, it emphasizes the expected clinical and epidemiological benefits of these approaches, including reduced virulence, disruption of biofilms, restoration of antibiotic susceptibility, and mitigation of resistance development through lower selective pressure.

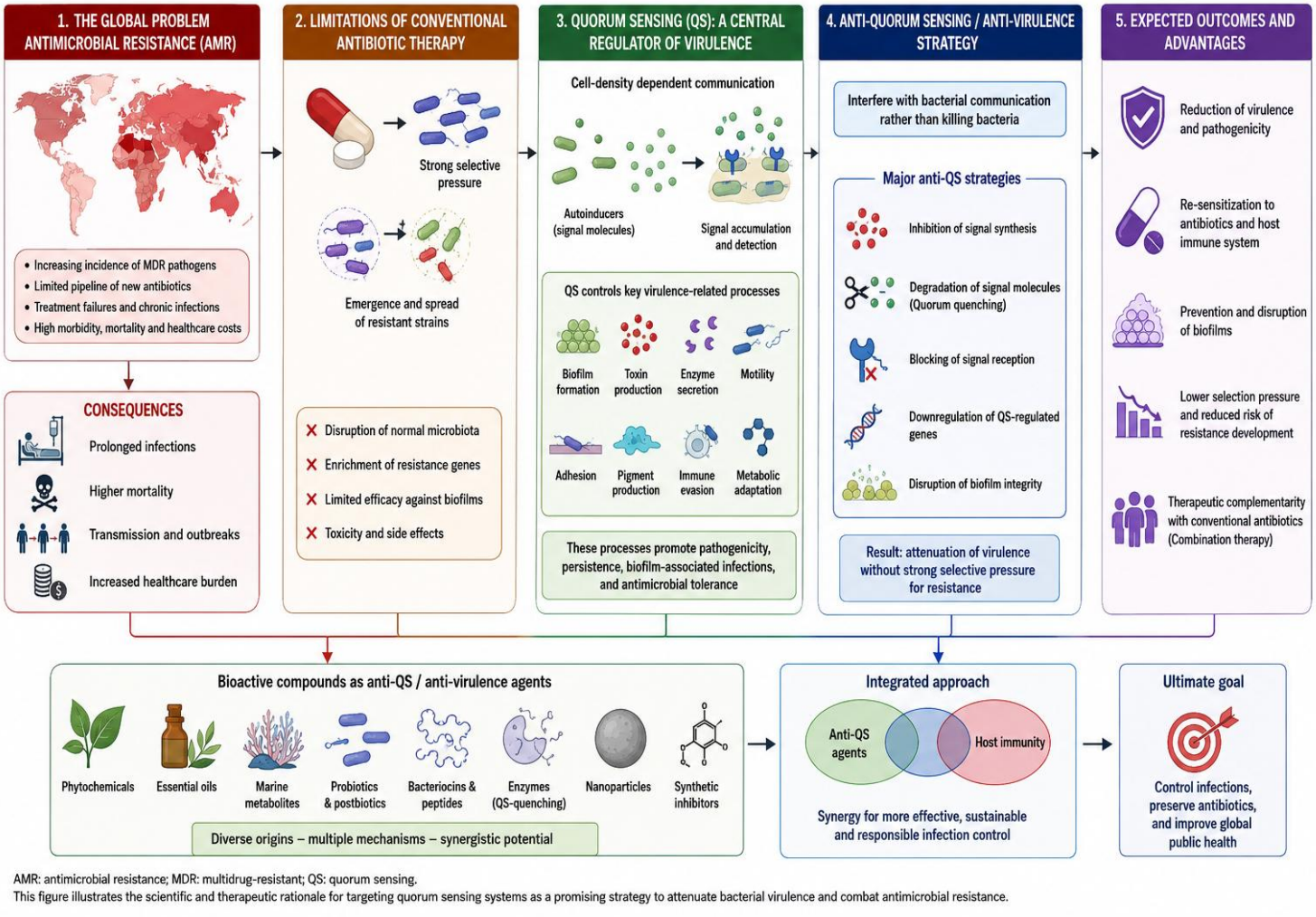

Figure 1. Global rationale for anti-quorum sensing and anti-virulence strategies against antimicrobial resistance.

2. Quorum Sensing Systems in Pathogenic Bacteria

Quorum sensing (QS) is a highly conserved bacterial communication process that enables microorganisms to coordinate collective behaviors according to population density, environmental conditions, and community structure. Through this mechanism, bacteria produce, release, accumulate, detect, and respond to small signaling molecules commonly referred to as autoinducers. When these molecules reach a critical threshold concentration, they interact with specific receptors and activate regulatory cascades that modify gene expression at the population level (Chu et al., 2024; Warrier et al., 2021). This process

allows bacteria to behave not merely as isolated unicellular organisms but as coordinated communities capable of adapting to complex ecological and host-associated environments.

In pathogenic bacteria, QS plays a central role in the regulation of virulence. It allows bacterial populations to synchronize the production of virulence factors only when a sufficient number of cells is present, thereby increasing the efficiency of infection and reducing premature detection by host immune defenses (Warrier et al., 2021; Naga et al., 2024). QS-regulated traits include biofilm formation, motility, toxin production, extracellular enzyme secretion, pigment synthesis, siderophore production, adhesion, immune evasion, stress tolerance, and antimicrobial tolerance (Sharma et al., 2023; Juszczuk-Kubiak, 2024). For this reason, QS is now considered a major target for anti-virulence strategies, particularly in the context of chronic, polymicrobial, and biofilm-associated infections (Hetta et al., 2024; Dehbanipour & Ghalavand, 2022).

The general organization of quorum sensing systems in Gram-negative and Gram-positive bacteria is summarized in Figure 2.

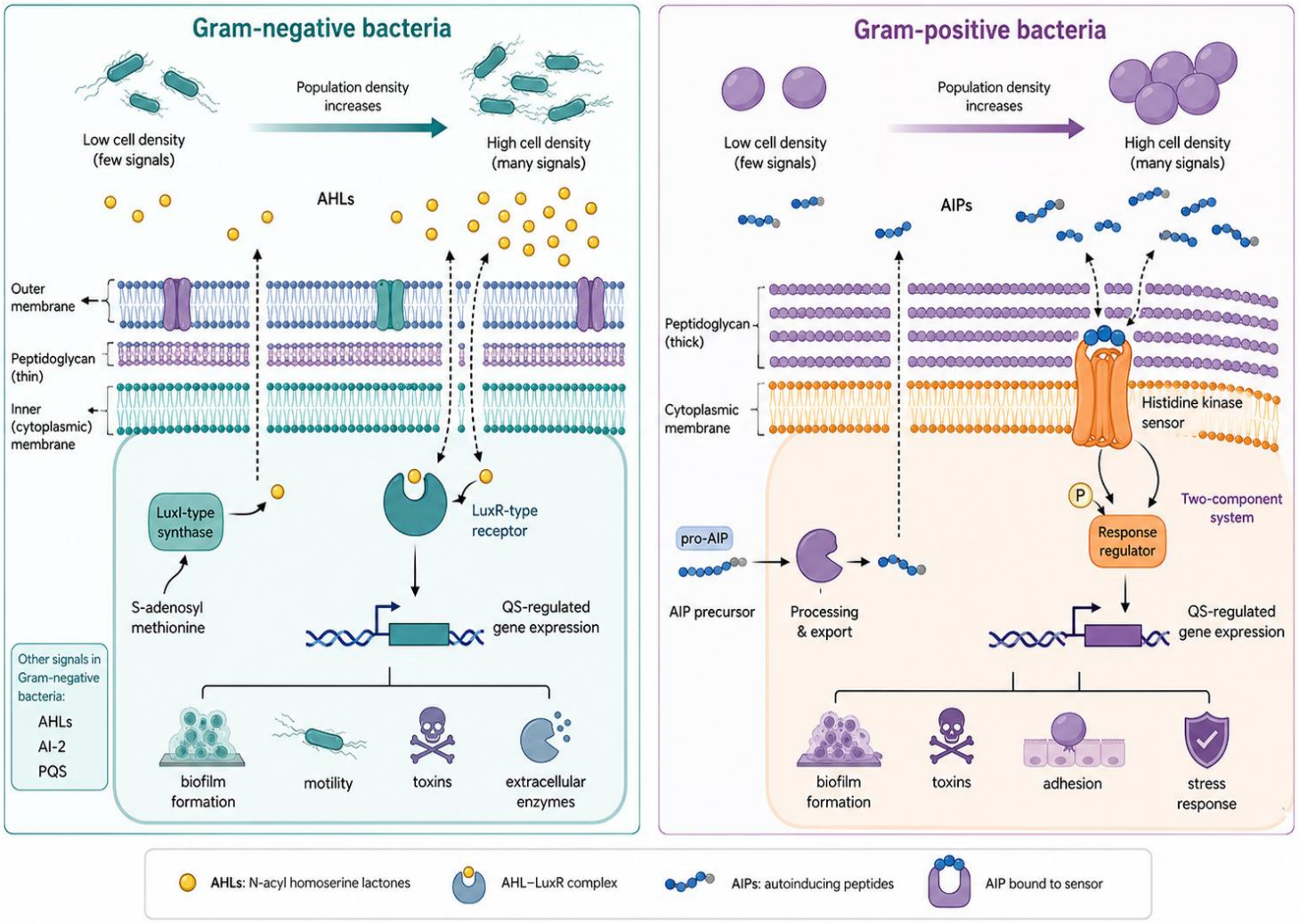

Figure 2. Overview of quorum sensing systems in Gram-positive and Gram-negative bacteria

2.1. Quorum sensing in Gram-negative bacteria

In Gram-negative bacteria, QS is most mediated by acyl-homoserine lactones (AHLs), although several other signaling molecules, including autoinducer-2 (AI-2), quinolone signals, diffusible signal factors, and indole-derived compounds, may also participate in bacterial communication (Chu et al., 2024). The classical model of Gram-negative QS is the LuxI/LuxR system. In this system, LuxI-type synthases produce AHL molecules, which diffuse across the bacterial membrane and accumulate in the extracellular environment as bacterial density increases. Once a threshold concentration is reached, AHLs bind to LuxR-type transcriptional regulators, leading to activation or repression of QS-controlled genes (Chu et al., 2024).

AHL molecules generally contain a conserved homoserine lactone ring and an acyl side chain that can vary in length, saturation, and substitution. These structural variations determine signal specificity, receptor affinity, diffusion capacity, and biological

activity. As a result, different bacterial species may use distinct AHL molecules to regulate species-specific behaviors, while structurally related signals may allow interspecies communication or interference in polymicrobial communities (Chu et al., 2024; Cui & Kim, 2024). This diversity is important because it explains why anti-QS compounds may have selective activity against some bacterial signaling systems while showing limited or no effects against others.

LuxI/LuxR-type systems regulate numerous virulence-associated phenotypes in Gram-negative pathogens. These include biofilm formation, surface motility, pigment production, protease secretion, siderophore production, oxidative stress adaptation, secretion systems, and host tissue damage (Warrier et al., 2021; Juszczuk-Kubiak, 2024). Therefore, inhibition of AHL synthesis, degradation of AHL molecules, blockade of LuxR-type receptors, or disruption of downstream QS-regulated transcription may reduce bacterial pathogenicity without necessarily affecting bacterial growth. This principle is central to the development of anti-QS and anti-virulence therapies (Hetta et al., 2024).

2.2. The Las, Rhl, and PQS systems in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is one of the most widely studied bacterial models for QS-regulated virulence. Its QS network is complex, hierarchical, and interconnected, involving mainly the Las, Rhl, and Pseudomonas quinolone signal (PQS) systems (Miranda et al., 2022; Zhang et al., 2025). The Las system includes LasI, which synthesizes N-3-oxo-dodecanoyl-homoserine lactone, and LasR, its cognate receptor. The Rhl system includes RhlI, which produces N-butanoyl-homoserine lactone, and RhlR, its transcriptional regulator. The PQS system is based on quinolone-derived signals and is regulated through PqsR/MvfR-dependent mechanisms (Miranda et al., 2022).

These systems regulate several major virulence factors, including pyocyanin, elastase, alkaline protease, rhamnolipids, hydrogen cyanide, lectins, siderophores, extracellular DNA release, motility, and biofilm development (Miranda et al., 2022; Zhang et al., 2025). The Las system is often considered an upper regulatory layer that influences both the Rhl and PQS systems, although this hierarchy may vary depending on strain background, nutrient availability, oxygen tension, infection stage, and environmental conditions (Miranda et al., 2022). This flexibility explains why QS responses in *P. aeruginosa* can differ substantially between planktonic cultures, biofilms, laboratory strains, and clinical isolates.

The importance of QS in *P. aeruginosa* is particularly evident in chronic infections, including cystic fibrosis lung infections, wound infections, urinary tract infections, and device-associated infections. In these settings, QS contributes to biofilm maturation, immune evasion, tissue damage, antimicrobial tolerance, and long-term persistence (Sharma et al., 2023; Zhang et al., 2025). However, chronic infection isolates may also display mutations in QS regulatory genes, leading to altered virulence profiles and phenotypic heterogeneity. Therefore, anti-QS studies using *P. aeruginosa* should not rely only on one laboratory strain or one virulence marker.

Methodologically, *P. aeruginosa* offers several measurable QS-regulated outputs, making it a valuable model for anti-QS screening. Pyocyanin quantification, elastase activity, rhamnolipid production, swimming, swarming and twitching motility, biofilm biomass, viable biofilm cell counts, and RT-qPCR analysis of *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, and *pqsR* are commonly used to evaluate the anti-QS activity of bioactive compounds (Miranda et al., 2022; Zhang et al., 2025). Nevertheless, a decrease in one phenotype does not necessarily prove direct QS inhibition. For example, reduced pyocyanin production may result from interference with QS, but it may also reflect growth inhibition, metabolic stress, oxidative imbalance, or altered precursor availability. Therefore, rigorous studies should combine phenotypic assays, gene expression analysis, and, when possible, direct quantification of QS signaling molecules.

2.3. AI-2-mediated quorum sensing and interspecies communication

Autoinducer-2 (AI-2) is frequently described as an interspecies bacterial signal because it is produced by a wide range of Gram-negative and Gram-positive bacteria. AI-2 is synthesized through the LuxS enzyme, which is involved in the activated methyl cycle. This dual role means that LuxS-related effects may reflect both communication and metabolic regulation, making interpretation more complex than in classical AHL-based systems (Chu et al., 2024; Juszczuk-Kubiak, 2024).

AI-2-mediated communication is particularly important in polymicrobial communities, where bacterial species interact through signaling molecules, metabolites, enzymes, and stress-response pathways. In such communities, QS may regulate biofilm architecture, cooperation, competition, virulence expression, antimicrobial tolerance, and spatial organization (Cui & Kim, 2024). This is highly relevant in chronic wounds, oral biofilms, respiratory infections, gastrointestinal infections, diabetic foot ulcers, and device-associated infections, where pathogens rarely exist as isolated monocultures.

In anti-QS research, AI-2 systems require careful methodological interpretation. Inhibition of LuxS-dependent phenotypes may indicate disruption of interspecies communication, but it may also reflect broader changes in bacterial metabolism. Therefore, studies investigating AI-2-related anti-QS activity should ideally combine phenotypic assays, AI-2 reporter systems, gene expression analysis, and evaluation of bacterial growth and viability. This is particularly important when testing complex bioactive substances such as plant extracts, essential oils, postbiotics, and nanoparticles, which may affect both signaling and metabolism.

2.4. Quorum sensing in Enterobacteriaceae

Members of the Enterobacteriaceae family, including *Escherichia coli* and *Klebsiella pneumoniae*, possess QS or QS-like regulatory systems that contribute to virulence, biofilm formation, adhesion, stress adaptation, and antimicrobial tolerance. Unlike many Gram-negative bacteria, *E. coli* does not usually produce AHLs through a classical LuxI homolog, but it can detect AHL molecules produced by other bacterial species through SdiA, a LuxR-family receptor (Chu et al., 2024). This enables *E. coli* to respond to chemical signals produced in polymicrobial environments.

In addition to SdiA-mediated signaling, *E. coli* uses AI-2/LuxS-related communication and indole-associated signaling to regulate biofilm formation, motility, adhesion, stress responses, and virulence-associated traits (Juszczuk-Kubiak, 2024). In pathogenic *E. coli* lineages, including enterohemorrhagic, enteropathogenic, and uropathogenic strains, QS-related regulation may influence colonization, toxin expression, epithelial attachment, and persistence. Consequently, anti-QS studies involving *E. coli* should consider that observed effects may involve multiple regulatory pathways rather than one classical QS circuit.

Klebsiella pneumoniae is another clinically important Enterobacteriaceae member in which QS-related mechanisms contribute to biofilm formation, capsule-associated virulence, fimbrial adhesion, siderophore production, and antimicrobial tolerance (Juszczuk-Kubiak, 2024; Cui & Kim, 2024). Because *K. pneumoniae* infections are strongly associated with hospital settings, urinary catheters, ventilator-associated pneumonia, and bloodstream infections, its ability to form biofilms and persist on medical devices is clinically significant. Anti-QS investigations in *K. pneumoniae* should therefore include biofilm assays, adhesion assays, capsule-related phenotypes, siderophore production, and expression analysis of virulence-associated genes.

2.5. Quorum sensing in *Acinetobacter baumannii*

Acinetobacter baumannii is an opportunistic Gram-negative pathogen of major clinical concern because of its capacity to survive in hospital environments, acquire multidrug resistance, and cause severe infections, including ventilator-associated pneumonia, bloodstream infections, wound infections, and device-associated infections. QS in *A. baumannii* is commonly associated with the AbaI/AbaR system, which resembles the LuxI/LuxR AHL-based system (Sun et al., 2021; Pumirat et al., 2024).

AbaI synthesizes AHL signals, while AbaR functions as the corresponding regulatory receptor. This system contributes to biofilm formation, surface-associated growth, motility, virulence, stress adaptation, and antibiotic susceptibility (Sun et al., 2021; Pumirat et al., 2024). Because *A. baumannii* can persist on abiotic surfaces and medical equipment, QS-regulated biofilm development is especially important for its pathogenic success in healthcare environments.

Anti-QS approaches targeting *A. baumannii* may aim to reduce biofilm biomass, impair adhesion, decrease motility, disrupt AHL-mediated signaling, or modulate expression of *abaI*, *abaR*, and biofilm-associated genes (Sun et al., 2021; Pumirat et al., 2024). However, as with other pathogens, reductions in biofilm formation must be interpreted in relation to bacterial growth, viability, and metabolic activity. A true anti-QS effect should not be concluded solely from reduced biomass unless supported by sub-MIC testing, signal interference assays, and molecular evidence.

2.6. Quorum sensing in Gram-positive bacteria

In Gram-positive bacteria, QS systems are generally mediated by autoinducing peptides (AIPs) rather than AHLs. These peptides are synthesized as precursor molecules, processed, exported, and detected by membrane-bound sensor kinases. Signal perception usually activates two-component regulatory systems composed of a sensor histidine kinase and a response regulator, which then modulate transcription of target genes (Williams, 2025; Yamazaki et al., 2024). This peptide-based communication regulates virulence, competence, bacteriocin production, biofilm formation, stress responses, and population-level adaptation.

The peptide nature of Gram-positive QS creates important biological differences from Gram-negative QS. AIPs are often highly specific, and small structural differences may determine whether a signal activates or inhibits a given receptor. This

specificity is especially important in *Staphylococcus aureus*, where different agr specificity groups may interfere with one another (Williams, 2025). Such cross-inhibition can influence strain competition, colonization, virulence expression, and disease outcome.

2.7. The agr system in *Staphylococcus aureus*

The accessory gene regulator, or agr system, is the best-characterized QS system in *Staphylococcus aureus*. It is one of the most clinically relevant peptide-based QS networks in Gram-positive pathogens. The agr locus generally contains *agrB*, *agrD*, *agrC*, and *agrA*. AgrD encodes the precursor peptide, AgrB processes and exports the mature AIP, AgrC functions as the membrane sensor kinase, and AgrA acts as the response regulator (Yamazaki et al., 2024; Williams, 2025).

When the extracellular concentration of AIP reaches a critical threshold, AIP activates AgrC, leading to phosphorylation of AgrA. Activated AgrA stimulates transcription from the P2 and P3 promoters, resulting in amplification of the agr system and production of RNAIII, a major regulatory RNA responsible for controlling numerous virulence-associated genes (Yamazaki et al., 2024). Through this system, *S. aureus* coordinates the transition from colonization to invasive infection.

At low cell density, *S. aureus* tends to express surface adhesins that promote attachment to host tissues and abiotic surfaces. At high cell density, agr activation represses several surface-associated proteins and promotes the production of secreted virulence factors, including hemolysins, proteases, lipases, phenol-soluble modulins, and immune-modulatory proteins (Yamazaki et al., 2024; Williams, 2025). This regulatory switch allows *S. aureus* to move from an adhesive colonizing state toward a tissue-damaging and disseminating phenotype.

The relationship between agr activity and biofilm formation is complex. In many contexts, agr activation promotes biofilm dispersal through increased production of proteases and surfactant-like peptides, whereas agr dysfunction may favor stable biofilm accumulation (Yamazaki et al., 2024). However, this relationship depends on strain background, infection site, biofilm maturity, environmental conditions, and nutrient availability. Therefore, anti-QS studies involving *S. aureus* must clearly define whether they are targeting toxin production, biofilm formation, biofilm dispersal, adhesion, or invasive virulence.

Methodologically, agr activity can be evaluated by hemolysis assays, δ-hemolysin detection, RNAIII quantification, reporter strains, RT-qPCR analysis of *agrA*, *agrC*, *hla*, *psm*, and RNAIII, and direct quantification of secreted toxins (Yamazaki et al., 2024; Williams, 2025). However, as in Gram-negative models, inhibition of one phenotype does not necessarily prove direct QS interference. Reduced hemolysis, for example, may result from agr inhibition, altered growth, toxin neutralization, membrane disruption, or metabolic stress.

2.8. Quorum sensing in *Enterococcus faecalis*

Enterococcus faecalis is an opportunistic Gram-positive pathogen associated with endodontic infections, urinary tract infections, wound infections, bacteremia, infective endocarditis, and hospital-acquired infections. Its pathogenicity is strongly linked to persistence under stress, biofilm formation, antimicrobial tolerance, and production of extracellular virulence factors (Yang et al., 2024). QS and peptide-mediated communication systems contribute to several of these traits.

In *E. faecalis*, peptide signaling can influence biofilm development, conjugation, bacteriocin production, gelatinase activity, aggregation, adhesion, and virulence gene regulation (Yang et al., 2024). Gelatinase, encoded by *gelE*, is often considered an important QS-associated virulence factor because it contributes to tissue damage, biofilm architecture, and immune evasion. Therefore, anti-QS studies involving *E. faecalis* commonly evaluate biofilm biomass, viable biofilm cells, gelatinase activity, aggregation, adhesion, and expression of virulence-associated genes.

Because *E. faecalis* is highly persistent in root canal infections and medical device-associated biofilms, it is a relevant model for studying bioactive compounds with anti-biofilm and anti-QS potential. However, methodological caution is necessary because reduced gelatinase activity or biofilm formation may reflect direct antimicrobial effects, changes in growth kinetics, or interference with general metabolism rather than specific disruption of QS-regulated pathways.

2.9. Quorum sensing in *Streptococcus* species

Streptococci use peptide-based QS systems to regulate genetic competence, bacteriocin production, biofilm formation, acid tolerance, stress adaptation, and virulence. In *Streptococcus mutans*, QS contributes to dental biofilm development, cariogenicity, aciduric behavior, bacteriocin production, and ecological competition within oral biofilms (Gao et al., 2024). This makes *S. mutans* an important model for anti-QS studies in oral microbiology and dental research.

Oral biofilms are not simple single-species systems. They are complex polymicrobial communities shaped by pH gradients, nutrient availability, oxygen tension, salivary components, host factors, and microbial interactions. QS contributes to community organization, metabolic cooperation, competition, and biofilm maturation (Gao et al., 2024; Cui & Kim, 2024). Therefore, anti-QS compounds targeting oral pathogens should ideally be evaluated in both monoculture and multispecies biofilm models.

In *Streptococcus pneumoniae*, peptide-based QS systems regulate competence and may influence virulence, adaptation, and genetic exchange. Although competence regulation is not always directly equivalent to virulence regulation, natural transformation can contribute to genetic diversity, capsule switching, and antimicrobial resistance acquisition. Thus, QS-related pathways in streptococci can influence both pathogenicity and evolutionary adaptation.

2.10. Quorum sensing in *Vibrio cholerae* and other vibrios

Vibrio cholerae represents another important model of QS-regulated virulence. Unlike bacteria in which high cell density activates virulence factor expression, *V. cholerae* displays a regulatory pattern in which QS can repress certain virulence traits at high cell density while promoting detachment and dissemination (Sajeevan et al., 2025). This illustrates an essential point: QS does not always function as a simple "on switch" for virulence. Its effects depend on bacterial species, ecological context, infection stage, and the specific regulatory architecture involved.

In *V. cholerae*, QS influences biofilm formation, virulence gene expression, host colonization, environmental persistence, and transmission dynamics (Sajeevan et al., 2025). This dual role is biologically important because *V. cholerae* must alternate between aquatic environments and the human host. QS helps coordinate this transition by regulating attachment, biofilm formation, toxin expression, and dispersal. Therefore, anti-QS strategies against vibrios must account for the possibility that QS inhibition may produce different outcomes depending on whether the target is colonization, toxin production, biofilm formation, or environmental survival.

2.11. Quorum sensing, biofilms, and antimicrobial tolerance

Biofilm formation is one of the most clinically important QS-associated phenotypes. Biofilms are structured microbial communities embedded in an extracellular matrix composed of polysaccharides, proteins, lipids, extracellular DNA, and other biomolecules. This matrix protects bacteria from antibiotics, disinfectants, immune responses, oxidative stress, nutrient limitation, and environmental fluctuations (Sharma et al., 2023; Juszczuk-Kubiak, 2024). QS contributes to several stages of biofilm development, including initial adhesion, microcolony formation, matrix production, maturation, and dispersal.

Within biofilms, QS can regulate extracellular polymeric substance production, motility, nutrient acquisition, secretion systems, persister cell formation, extracellular DNA release, and community-level stress responses (Warrier et al., 2021; Sharma et al., 2023). This makes QS particularly relevant to chronic infections, catheter-associated infections, dental plaque, implant-associated infections, respiratory infections, and wound infections. Biofilm-associated bacteria often display increased tolerance to antimicrobial agents, not necessarily because they are genetically resistant, but because the biofilm lifestyle creates physiological and structural protection.

QS may also indirectly contribute to antimicrobial resistance and tolerance by regulating biofilm formation, efflux activity, stress responses, and interspecies cooperation in polymicrobial infections (Cui & Kim, 2024). In polymicrobial biofilms, one species may protect another through enzyme production, matrix reinforcement, metabolic cooperation, or modification of the local microenvironment. These interactions can reduce antibiotic penetration, enhance survival, and complicate treatment outcomes.

2.12. Quorum sensing and polymicrobial infections

Many clinically relevant infections are polymicrobial rather than monospecies. Chronic wounds, diabetic foot ulcers, dental plaque, cystic fibrosis lung infections, otitis media, gastrointestinal infections, and device-associated infections often contain multiple bacterial species that interact through QS signals, metabolites, extracellular enzymes, and stress-response mechanisms (Cui & Kim, 2024). In such contexts, QS may coordinate cooperation, competition, spatial organization, biofilm maturation, virulence expression, and antimicrobial tolerance.

Polymicrobial QS has major methodological implications. A compound that inhibits QS in a single-species culture may behave differently in a multispecies community. It may suppress one pathogen while indirectly favoring another, disrupt harmful

communication while preserving beneficial microbiota, or alter community structure in unexpected ways (Cui & Kim, 2024). Therefore, anti-QS research should progressively move beyond simple monoculture assays and include multispecies biofilm models, co-culture systems, microbiome-compatible assays, and omics-based community analyses.

2.13. Quorum sensing as a target for anti-virulence therapy

The central role of QS in virulence regulation makes it an attractive target for anti-virulence therapy. Unlike conventional antibiotics, which aim to kill bacteria or inhibit their growth, anti-QS strategies seek to disarm pathogens by interfering with communication and virulence regulation (Dehbanipour & Ghalavand, 2022; Hetta et al., 2024). This approach may reduce the selective pressure associated with bactericidal or bacteriostatic agents and may preserve beneficial microbiota.

Anti-QS compounds may act through several mechanisms: inhibition of autoinducer synthesis, enzymatic degradation of signaling molecules, chemical inactivation of signals, receptor antagonism, inhibition of signal transduction, modulation of QS-regulated gene expression, or suppression of downstream virulence phenotypes (Naga et al., 2024; Hetta et al., 2024). Bioactive compounds such as plant metabolites, essential oils, microbial metabolites, postbiotics, antimicrobial peptides, and nanoparticles may influence one or several of these steps.

However, because many bioactive compounds have pleiotropic effects, anti-QS activity must be demonstrated carefully. Plant extracts may contain antimicrobial, antioxidant, membrane-active, and enzyme-modulating molecules. Nanoparticles may affect bacteria through metal ion release, oxidative stress, membrane disruption, protein interaction, or biofilm matrix destabilization. Postbiotics may contain organic acids, peptides, biosurfactants, enzymes, and signaling-interfering metabolites. Therefore, a reduction in QS-related virulence factors does not automatically prove direct QS inhibition.

For this reason, rigorous anti-QS studies should integrate several levels of evidence: determination of MIC and sub-MIC concentrations, confirmation that tested concentrations do not significantly inhibit growth, evaluation of multiple QS-regulated phenotypes, measurement of signaling molecules when possible, analysis of QS-related gene expression, imaging of biofilm architecture, and validation in biologically relevant in vivo or ex vivo models (Naga et al., 2024; Hetta et al., 2024). This integrated methodological approach is essential for distinguishing true quorum sensing inhibition from general antibacterial or antibiofilm effects.

2.14. Synthesis

Overall, QS systems in pathogenic bacteria are diverse, dynamic, and deeply integrated into virulence regulation. Gram-negative bacteria commonly use AHLs, AI-2, quinolone signals, and other diffusible molecules, whereas Gram-positive bacteria mainly rely on autoinducing peptides and two-component regulatory systems (Chu et al., 2024; Williams, 2025). Major pathogens such as *P. aeruginosa*, *S. aureus*, *A. baumannii*, *E. coli*, *K. pneumoniae*, *E. faecalis*, *S. mutans*, and *V. cholerae* use QS or QS-like pathways to coordinate biofilm formation, motility, toxin production, enzyme secretion, adhesion, immune evasion, antimicrobial tolerance, and persistence (Miranda et al., 2022; Yamazaki et al., 2024; Sun et al., 2021; Gao et al., 2024; Sajeevan et al., 2025).

For a methodological review, this biological complexity justifies the need for standardized, integrated, and critically interpreted experimental approaches. Understanding QS systems is essential before evaluating the effects of bioactive compounds on QS-related virulence factors. Without this mechanistic foundation, anti-QS studies risk confusing growth inhibition with virulence attenuation, antibiofilm activity with signal disruption, or nonspecific toxicity with targeted quorum sensing interference. Therefore, the following sections will examine the major classes of bioactive compounds investigated as anti-QS agents and the methodological frameworks required to evaluate their effects rigorously.

Given the remarkable diversity of quorum sensing systems among clinically relevant pathogens, a comparative overview is necessary to understand the signaling molecules involved, their regulatory components, the virulence traits under their control, and their relevance as targets for anti-quorum sensing interventions. The major quorum sensing systems described in pathogenic bacteria are summarized in Table 1.

Table 1. Major quorum sensing systems in pathogenic bacteria

Bacterial group / species	Major QS system(s)	Main autoinducer / signal molecule(s)	Key synthase / sensor / regulator components	Major QS-regulated virulence traits	Relevance for anti-QS research	References
Pseudomonas aeruginosa	Las, Rhl, PQS systems	3-oxo-C12-HSL, C4-HSL, PQS, HHQ	LasI/LasR, RhlI/RhlR, PqsABCDE/PqsR/MvfR	Biofilm formation, elastase, proteases, pyocyanin, rhamnolipids, motility, immune evasion, antimicrobial tolerance	One of the most extensively studied QS models; highly relevant for anti-biofilm and anti-virulence strategies	Miranda et al., 2022; Zhang et al., 2025; Gad et al., 2024
Staphylococcus aureus	Agr system	Autoinducing peptides	AgrD/AgrB, AgrC/AgrA, RNAIII	Hemolysins, proteases, lipases, phenol-soluble modulins, toxin production, biofilm dispersal, immune evasion	Central model for peptide-based QS inhibition in Gram-positive pathogens	Yamazaki et al., 2024; Williams, 2025; Podkowik et al., 2024
Vibrio cholerae	LuxO/HapR-dependent QS network	CAI-1, AI-2	CqsA/CqsS, LuxS/LuxPQ, LuxO, HapR	Biofilm formation, virulence regulation, motility, intestinal colonization, environmental persistence, dissemination	Important model for multi-signal QS integration and infection-stage-dependent virulence regulation	Sajeevan et al., 2025; Chu et al., 2024
Vibrio harveyi / *Vibrio campbellii*	LuxM/LuxN, LuxS/LuxPQ, CqsA/CqsS systems	HAI-1, AI-2, CAI-1	LuxM/LuxN, LuxS/LuxPQ, CqsA/CqsS, LuxO, LuxR	Bioluminescence, biofilm formation, motility, secretion-associated virulence behaviors	Classical biosensor model for QS signal detection and anti-QS screening	Chu et al., 2024; Warrier et al., 2021
Vibrio vulnificus	LuxS/AI-2 and SmcR-associated QS	AI-2 and QS-associated signals	LuxS, LuxO, SmcR	Cytotoxicity, metalloprotease production, motility, biofilm formation, host invasion	Relevant for foodborne, marine-associated and opportunistic infections	Chu et al., 2024; Ding et al., 2024
Escherichia coli	AI-2/LuxS system; SdiA-mediated interspecies signaling	AI-2; exogenous AHL sensing through SdiA	LuxS, Lsr system, SdiA	Biofilm formation, motility, adhesion, acid tolerance, stress response, virulence gene modulation	Useful model for interspecies QS, gut colonization, polymicrobial signaling and biofilm regulation	Chu et al., 2024; Juszczuk-Kubiak, 2024; Cui & Kim, 2024
Enterohemorrhagic *E. coli*	AI-3/catecholamine-related sensing; AI-2-related pathways	AI-3-like signals, epinephrine/norepinephrine, AI-2	QseC/QseB, QseE/QseF, LuxS-associated pathways	Motility, Shiga toxin regulation, type III secretion, epithelial attachment	Important for host–pathogen signaling and anti-virulence targeting	Chu et al., 2024; Juszczuk-Kubiak, 2024
Salmonella enterica	LuxS/AI-2; SdiA-mediated AHL sensing	AI-2, exogenous AHLs	LuxS, Lsr system, SdiA	Biofilm formation, motility, adhesion, invasion, stress adaptation	Relevant for foodborne infection, biofilm persistence and interspecies communication	Chu et al., 2024; Ding et al., 2024; Cui & Kim, 2024
Klebsiella pneumoniae	AI-2/LuxS-associated QS and QS-like regulation	AI-2 and QS-associated signals	LuxS-associated pathways; biofilm- and virulence-associated regulators	Biofilm formation, capsule-associated virulence, fimbrial adhesion, siderophore production, antimicrobial tolerance	Clinically important model for hospital-associated infections, device-associated biofilms and MDR persistence	Juszczuk-Kubiak, 2024; Cui & Kim, 2024; Zhang et al., 2025

Acinetobacter baumannii	AbaI/AbaR system	3-hydroxy-C12-HSL and related AHLs	AbaI, AbaR	Biofilm formation, surface motility, adhesion, persistence, virulence, antibiotic susceptibility modulation	Emerging target in multidrug-resistant hospital-associated infections	Sun et al., 2021; Pumirat et al., 2024
Burkholderia cepacia complex	CepIR and related LuxIR-type systems	C8-HSL, C6-HSL and related AHLs	CepI/CepR, CciI/CciR, BviI/BviR depending on species	Biofilm formation, protease production, siderophore production, motility, chronic infection adaptation	Relevant in cystic fibrosis and opportunistic respiratory infections	Chu et al., 2024; Warrier et al., 2021; Cui & Kim, 2024
Serratia marcescens	SwrIR, SmaIR systems	C4-HSL, C6-HSL and related AHLs	SwrI/SwrR, SmaI/SmaR	Swarming motility, prodigiosin production, proteases, biofilm formation	Useful for pigment- and motility-based anti-QS screening	Chu et al., 2024; Warrier et al., 2021
Chromobacterium violaceum	CviIR system	C6-HSL and related AHLs	CviI, CviR	Violacein production, biofilm formation, protease production, cyanide production, virulence	Widely used model organism for preliminary screening of QS inhibitors	Warrier et al., 2021; Naga et al., 2024; Hetta et al., 2024
Aeromonas hydrophila	AhyIR system; LuxS/AI-2	C4-HSL, C6-HSL, AI-2	AhyI/AhyR, LuxS	Biofilm formation, motility, hemolysin, protease, lipase, enterotoxin production	Relevant for aquatic, foodborne and opportunistic infections	Chu et al., 2024; Ding et al., 2024; Warrier et al., 2021
Yersinia pseudotuberculosis / *Yersinia enterocolitica*	YpsIR / YenIR systems	AHLs, including C6-HSL and related molecules	YpsI/YpsR, YenI/YenR	Motility, aggregation, biofilm formation, secretion-associated virulence traits	Useful for studying QS control of motility and host-associated persistence	Chu et al., 2024; Warrier et al., 2021
Clostridioides difficile	Agr-like system; LuxS/AI-2	Autoinducing peptides, AI-2	AgrD/AgrB/AgrC/AgrA-like components, LuxS	Toxin production, sporulation-associated regulation, biofilm formation, colonization	Important for toxin regulation and intestinal infection models	Williams, 2025; Juszczuk-Kubiak, 2024; Ding et al., 2024
Enterococcus faecalis	Fsr system	Gelatinase biosynthesis-activating pheromone	FsrD/FsrB, FsrC/FsrA	Gelatinase production, serine protease activity, biofilm formation, tissue damage, endocarditis-related virulence	Relevant model for Gram-positive peptide QS and enzyme-mediated virulence	Yang et al., 2024; Williams, 2025
Streptococcus pneumoniae	ComCDE competence system; LuxS/AI-2	Competence-stimulating peptide, AI-2	ComC/ComD/ComE, LuxS	Competence, biofilm formation, fratricide, colonization, virulence adaptation	Important for competence-associated virulence, genetic exchange and adaptation	Williams, 2025; Juszczuk-Kubiak, 2024
Streptococcus mutans	ComCDE, ComRS, LuxS/AI-2	CSP, XIP, AI-2	ComC/ComD/ComE, ComR/ComS, LuxS	Dental biofilm formation, acid tolerance, bacteriocin production, competence, cariogenicity	Highly relevant for oral biofilms, dental caries and stomatology-related anti-QS studies	Gao et al., 2024; Cui & Kim, 2024
Listeria monocytogenes	Agr-like system; LuxS/AI-2	Autoinducing peptides, AI-2	AgrBDCA-like system, LuxS	Biofilm formation, adhesion, invasion, stress adaptation, food-processing persistence	Relevant for food safety, surface persistence and anti-adhesion approaches	Ding et al., 2024; Juszczuk-Kubiak, 2024
Helicobacter pylori	LuxS/AI-2-related signaling	AI-2	LuxS and chemoreception-associated pathways	Motility, biofilm formation, colonization, stress adaptation	Relevant for gastric colonization and persistence	Juszczuk-Kubiak, 2024; Warrier et al., 2021

3. Bioactive Compounds Targeting Quorum Sensing Systems

Bioactive compounds targeting quorum sensing (QS) systems represent an increasingly important class of anti-virulence agents because they aim to attenuate bacterial pathogenicity without necessarily killing bacterial cells. Unlike conventional antibiotics, which mainly act by inhibiting bacterial growth or viability, anti-QS agents interfere with communication-dependent regulation of virulence factors, including biofilm formation, motility, toxin production, pigment synthesis, extracellular enzyme secretion, adhesion, and immune evasion (Hetta et al., 2024; Naga et al., 2024). This strategy is particularly attractive in the context of multidrug-resistant bacterial infections because it may reduce virulence expression while potentially exerting lower selective pressure than bactericidal or bacteriostatic drugs (Hetta et al., 2024; Bhuiyan et al., 2025).

The mechanisms through which bioactive compounds interfere with QS are diverse. They may inhibit autoinducer synthesis, degrade or chemically inactivate signaling molecules, block signal receptors, disrupt signal transduction, suppress QS-regulated gene expression, or indirectly disturb QS-controlled phenotypes such as biofilm development and toxin production (D'Aquila et al., 2024; Hetta et al., 2024; Sikdar & Elias, 2020). However, because many bioactive compounds also possess antibacterial, antibiofilm, antioxidant, membrane-active, or metabolic effects, their classification as true QS inhibitors requires careful methodological validation.

The diversity of bioactive compounds investigated as QS modulators is considerable. These include plant-derived metabolites, essential oils, marine natural products, microbial metabolites, probiotics, postbiotics, biosurfactants, bacteriocins, antimicrobial peptides, quorum-quenching enzymes, synthetic analogues, and nanoparticles (Alum et al., 2025; Papaneophytou, 2026; Hu et al., 2024). This chemical and biological diversity provides a broad reservoir of anti-virulence candidates, but it also creates major challenges related to reproducibility, standardization, chemical characterization, mechanism identification, and translation toward clinical or industrial applications (Papaneophytou, 2026; Naga et al., 2024).

The major classes of bioactive compounds investigated as quorum sensing inhibitors and anti-virulence agents are summarized in Figure 3.

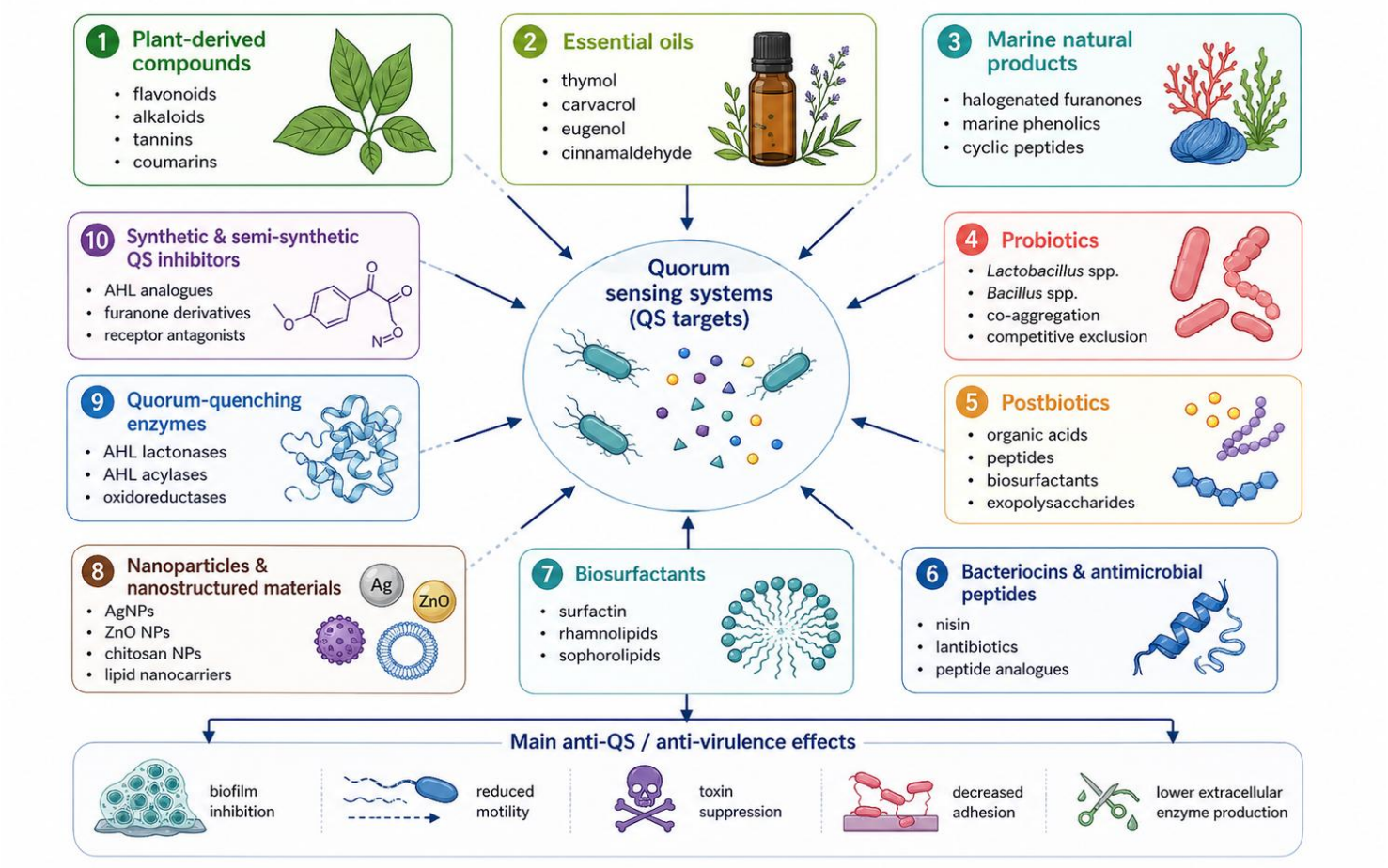

Figure 3. Major classes of bioactive compounds targeting quorum sensing systems

3.1. Plant-derived bioactive compounds

Plant-derived compounds are among the most widely investigated natural inhibitors of QS-regulated virulence. Medicinal plants produce a broad spectrum of secondary metabolites, including phenolic acids, flavonoids, tannins, alkaloids, terpenoids, coumarins, quinones, saponins, and essential oil constituents, many of which have been reported to interfere with bacterial communication systems (Papaneophytou, 2026; Alum et al., 2025). Their interest lies not only in their antimicrobial activity but also in their ability to attenuate virulence phenotypes at concentrations below those required to inhibit bacterial growth.

Phytochemicals may affect QS through several mechanisms. Some compounds can structurally mimic bacterial autoinducers and compete for receptor binding, while others may inhibit signal synthesis, destabilize signaling molecules, downregulate QS receptor expression, interfere with transcriptional regulators, or suppress downstream virulence factor production (Papaneophytou, 2026; Helcman et al., 2025). Because plant-derived compounds often act on multiple biological targets, their anti-QS effects should not be concluded from a single phenotypic observation. Instead, strong evidence requires sub-MIC testing, growth controls, biosensor assays, virulence phenotype evaluation, and molecular confirmation.

3.1.1. Phenolic compounds and flavonoids

Phenolic compounds and flavonoids constitute one of the most important groups of plant-derived QS inhibitors. Molecules such as quercetin, luteolin, kaempferol, naringenin, apigenin, catechin, gallic acid, caffeic acid, ferulic acid, chlorogenic acid, and cinnamic acid derivatives have been associated with reduced QS-regulated virulence in several pathogenic bacteria (Papaneophytou, 2026; Helcman et al., 2025). These compounds may interfere with QS receptors, reduce AHL-mediated signaling, suppress virulence gene expression, and attenuate phenotypes such as biofilm formation, pigment production, motility, protease activity, and toxin secretion.

In *Pseudomonas aeruginosa*, phenolic compounds and flavonoids are frequently evaluated through pyocyanin quantification, elastase activity, rhamnolipid production, motility assays, biofilm biomass measurement, and expression analysis of *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, and *pqsR* (Hetta et al., 2024; Papaneophytou, 2026). However, these molecules may also influence oxidative balance, iron availability, membrane permeability, and bacterial metabolism. Therefore, reductions in QS-related virulence factors must be interpreted alongside growth kinetics, viability assays, pH control, and solvent controls.

3.1.2. Tannins, alkaloids, terpenoids, and coumarins

Tannins are polyphenolic compounds capable of interacting with proteins, enzymes, polysaccharides, and bacterial surfaces. Their anti-QS and antibiofilm activities may involve inhibition of adhesion, disruption of extracellular polymeric substances, modulation of enzyme activity, and interference with QS-regulated phenotypes (Alum et al., 2025; Papaneophytou, 2026). However, tannins can also bind nonspecifically to assay components, which may interfere with colorimetric, enzymatic, protease, elastase, and hemolysis assays. This makes assay-interference controls particularly important.

Alkaloids represent another relevant class of plant-derived compounds with potential anti-QS effects. Several alkaloids have been associated with inhibition of biofilm formation, pigment production, motility, and virulence gene expression (Alum et al., 2025). Their mechanisms may include receptor antagonism, enzyme inhibition, modulation of bacterial regulatory pathways, and interference with nucleic acid or protein function. Because many alkaloids also possess direct antibacterial or cytotoxic activities, anti-QS interpretations must be supported by sub-inhibitory concentration testing and cytotoxicity evaluation.

Terpenoids and essential oil constituents such as thymol, carvacrol, eugenol, cinnamaldehyde, limonene, linalool, menthol, and geraniol have also been widely investigated as anti-QS and antibiofilm agents (Touati et al., 2025; Maggio et al., 2025; Srinivasan et al., 2025). These compounds may reduce bacterial adhesion, motility, biofilm formation, pigment production, toxin secretion, and extracellular enzyme release. Nevertheless, because many terpenoids are hydrophobic and membrane-active, their effects may result from membrane perturbation rather than specific QS inhibition. Therefore, membrane integrity assays, leakage assays, and growth controls are necessary.

Coumarins have attracted attention because some members of this class can interfere with QS-regulated virulence in Gram-negative bacteria. Their aromatic lactone structure may support interaction with QS receptors or signal-related enzymes (Papaneophytou, 2026). In methodological studies, coumarins and related molecules may be screened using AHL biosensor strains, molecular docking against LasR, RhlR, or PqsR, and phenotypic assays in *P. aeruginosa*. However, docking predictions must be experimentally confirmed because predicted receptor binding alone does not prove biological QS inhibition.

3.1.3. Essential oils and complex plant extracts

Essential oils are complex mixtures of volatile bioactive compounds, mainly terpenes, terpenoids, and phenylpropanoids. They are widely studied for their antibacterial, antibiofilm, anti-QS, and antibiotic-potentiating effects (Touati et al., 2025; Maggio et al., 2025; Srinivasan et al., 2025). Essential oils may inhibit bacterial adhesion, reduce motility, suppress pigment production, decrease extracellular enzyme activity, alter biofilm architecture, and modulate QS-related gene expression.

However, essential oils present major reproducibility challenges. Their chemical composition may vary according to plant species, chemotype, geographical origin, harvesting season, extraction procedure, storage conditions, and analytical method (Touati et al., 2025). Therefore, studies on QS should include chemical characterization using GC-MS, HPLC, LC-MS, or related analytical approaches before biological interpretation. Without chemical profiling, it is difficult to compare results between studies or identify the molecules responsible for anti-QS activity.

Crude plant extracts present similar methodological difficulties. They may contain hundreds of compounds acting synergistically, additively, or antagonistically. While this complexity may be pharmacologically valuable, it complicates mechanistic interpretation. For this reason, plant extract studies should ideally use bioassay-guided fractionation, chemical identification of active fractions, retesting of purified or enriched fractions, and confirmation through phenotypic and molecular anti-QS assays (Papaneophytou, 2026; Alum et al., 2025).

3.2. Marine natural products

Marine organisms, including algae, sponges, corals, marine bacteria, fungi, and actinomycetes, represent important sources of anti-QS compounds. Marine ecosystems are highly competitive environments in which chemical signaling and chemical interference play major ecological roles. As a result, marine-derived metabolites may naturally function as QS modulators or quorum-quenching agents (Mazumder et al., 2024; Coolahan et al., 2025).

Halogenated furanones are among the most historically important marine-derived QS inhibitors. They have been reported to interfere with AHL-dependent QS by destabilizing LuxR-type regulators or competing with natural autoinducer signaling (D'Aquila et al., 2024; Sikdar & Elias, 2020). Although these compounds provide an important conceptual basis for anti-QS drug discovery, some derivatives may present toxicity, instability, or limited therapeutic suitability.

Other marine compounds, including cyclic peptides, diketopiperazines, brominated metabolites, marine phenolics, polysaccharides, and fungal secondary metabolites, have also been investigated for their ability to downregulate QS-regulated phenotypes (Mazumder et al., 2024; Alum et al., 2025). Their mechanisms may include receptor antagonism, inhibition of signal production, suppression of virulence gene expression, interference with adhesion, and disruption of biofilm formation. However, marine natural products often require careful purification, structural elucidation, toxicity assessment, and validation in infection-relevant models before they can be considered credible anti-QS candidates.

3.3. Microbial-derived compounds

Microorganisms themselves produce several compounds capable of modulating QS systems. These include metabolites from probiotics, postbiotics, bacteriocins, biosurfactants, organic acids, enzymes, peptides, and secondary metabolites from bacteria and fungi (Erdinc et al., 2025; Maftei et al., 2026). Such compounds are biologically relevant because microbes naturally compete through chemical communication, signal interference, nutrient competition, and secretion of antagonistic metabolites.

3.3.1. Probiotics

Probiotics may interfere with pathogen QS through multiple mechanisms, including secretion of antimicrobial metabolites, production of organic acids, competition for adhesion sites, release of biosurfactants, enzymatic degradation of signaling molecules, modulation of host immune responses, and alteration of local microbial ecology (Maftei et al., 2026). Some probiotic strains have been associated with reduced biofilm formation, decreased virulence gene expression, and attenuation of QS-regulated phenotypes in pathogenic bacteria.

However, the anti-QS activity of probiotics is highly strain-dependent. Live probiotic cells can affect pathogens through nutrient competition, acidification, bacteriocin production, hydrogen peroxide production, co-aggregation, and direct growth inhibition (Maftei et al., 2026; Erdinc et al., 2025). Therefore, anti-QS studies should distinguish between the effects of live cells, heat-killed cells, cell-free supernatants, neutralized supernatants, proteinase-treated supernatants, and purified

metabolites. Without these controls, it is difficult to determine whether virulence attenuation results from QS interference or from general antagonism.

3.3.2. Postbiotics

Postbiotics are preparations of inanimate microorganisms and/or their components that confer biological activity. In anti-QS research, postbiotics are particularly promising because they may contain secreted metabolites, organic acids, peptides, enzymes, biosurfactants, exopolysaccharides, cell wall components, and signaling-interfering molecules (Amobonye et al., 2025; Erdinc et al., 2025; Tomičić et al., 2025). These components may reduce pathogen adhesion, disrupt biofilm formation, suppress virulence gene expression, and interfere with QS-related phenotypes.

Postbiotic preparations are complex and depend strongly on microbial strain, culture medium, incubation time, growth phase, processing method, heat treatment, filtration, and storage conditions (Amobonye et al., 2025; Maftei et al., 2026). Postbiotic studies should then include detailed preparation protocols, medium controls, pH-neutralized controls, heat-treatment controls, enzyme-treatment controls, and chemical or metabolomic characterization. This is especially important because many postbiotic effects may be caused by organic acids or pH changes rather than direct QS inhibition.

Postbiotics may reduce QS-related virulence by inhibiting autoinducer accumulation, degrading signaling molecules, suppressing QS gene expression, destabilizing biofilm matrix, changing surface hydrophobicity, reducing motility, or interfering with adhesion (Tomičić et al., 2025; Erdinc et al., 2025). However, because their composition is heterogeneous, mechanistic claims should be made cautiously unless supported by fractionation, metabolite identification, and molecular validation.

3.3.3. Bacteriocins and antimicrobial peptides

Bacteriocins and antimicrobial peptides are increasingly investigated as anti-virulence and anti-QS agents. These molecules may act by disrupting bacterial membranes, penetrating biofilms, inhibiting signaling pathways, degrading or mimicking peptide signals, or antagonizing QS receptors (Khan et al., 2025). Their relevance is particularly strong in Gram-positive pathogens, where QS systems frequently rely on peptide autoinducers.

In *Staphylococcus aureus*, peptide analogues may interfere with the agr system by competing with natural autoinducing peptides and preventing activation of AgrC/AgrA-dependent signaling. This may reduce RNAIII expression, hemolysin production, phenol-soluble modulin expression, and other agr-regulated virulence factors (Khan et al., 2025; Hetta et al., 2024). In Gram-negative bacteria, antimicrobial peptides may reduce biofilm formation, destabilize bacterial communities, or interfere indirectly with QS-regulated phenotypes.

Despite their promise, peptide-based anti-QS agents face several translational limitations, including proteolytic degradation, limited stability, poor tissue penetration, possible immunogenicity, high production cost, and delivery difficulties (Khan et al., 2025). Nanocarriers, hydrogels, surface coatings, and peptide engineering may improve their stability and bioavailability.

3.3.4. Biosurfactants

Biosurfactants are amphiphilic microbial molecules capable of reducing surface tension and modifying bacterial adhesion, motility, and biofilm formation. They include rhamnolipids, lipopeptides, glycolipids, sophorolipids, surfactin, and other surface-active metabolites (Adnan et al., 2023; Khairunnisa et al., 2024). Their antibiofilm activity may result from reduced surface adhesion, disruption of biofilm architecture, alteration of cell surface hydrophobicity, increased membrane permeability, and interference with signaling gradients.

Biosurfactants derived from probiotic or environmental microorganisms may inhibit virulence factors of Gram-negative and Gram-positive pathogens, including biofilm formation, motility, and extracellular enzyme activity (Adnan et al., 2023; Khairunnisa et al., 2024). However, because biosurfactants can physically disrupt bacterial membranes or biofilm matrices, their effects should not automatically be interpreted as QS inhibition. To support anti-QS claims, studies should include QS reporter assays, signal molecule detection, gene expression analysis, and growth-independent phenotypic validation.

3.4. Nanoparticles and nanostructured materials

Nanoparticles and nanostructured materials have become increasingly important in anti-QS research. These include silver nanoparticles, gold nanoparticles, zinc oxide nanoparticles, copper oxide nanoparticles, titanium dioxide nanoparticles,

selenium nanoparticles, chitosan nanoparticles, polymeric nanoparticles, lipid nanoparticles, nanogels, and functionalized nanocarriers (Hu et al., 2024; Afrasiabi et al., 2024). Nanomaterials may regulate QS at several levels, including signal synthesis, signal secretion, signal accumulation, signal perception, receptor activation, signal transduction, and downstream virulence expression (Hu et al., 2024).

Silver nanoparticles are among the most widely studied nanomaterials in antimicrobial and anti-QS research. They may reduce biofilm formation, motility, pigment production, extracellular enzyme activity, and virulence gene expression in pathogenic bacteria (Hu et al., 2024; Afrasiabi et al., 2024). Proposed mechanisms include silver ion release, oxidative stress generation, membrane disruption, enzyme inhibition, DNA interaction, and interference with QS-regulated pathways. However, this multiplicity of mechanisms also complicates interpretation, because reduced virulence may result from general nanoparticle toxicity rather than selective QS inhibition.

Zinc oxide, copper oxide, selenium, and titanium dioxide nanoparticles may generate reactive oxygen species, interact with bacterial membranes, alter metabolism, and disrupt biofilm structure (Hu et al., 2024). Chitosan nanoparticles are attractive because chitosan is biocompatible, cationic, and able to interact with bacterial surfaces and extracellular polymeric substances. Lipid-based and polymeric nanoparticles may also serve as delivery systems for phytochemicals, peptides, enzymes, antibiotics, or QS inhibitors, improving stability, solubility, targeted delivery, and biofilm penetration.

For anti-QS studies, nanoparticles must be physicochemically characterized before biological interpretation. Essential parameters include particle size, morphology, surface charge, polydispersity index, crystallinity, elemental composition, functional groups, colloidal stability, dissolution behavior, and aggregation state in biological media (Hu et al., 2024; Afrasiabi et al., 2024). Techniques such as dynamic light scattering, zeta potential analysis, scanning electron microscopy, transmission electron microscopy, X-ray diffraction, Fourier-transform infrared spectroscopy, UV–visible spectroscopy, and inductively coupled plasma analysis may be required depending on the nanomaterial studied.

Nanoparticle studies should also include appropriate controls, including precursor controls, reducing agent controls, coating controls, vehicle controls, ion release controls, and particle-free supernatant controls (Hu et al., 2024). Without such controls, it is difficult to determine whether anti-QS activity is due to the nanoparticle itself, dissolved ions, residual synthesis reagents, surface coatings, or medium-dependent transformations.

3.5. Quorum-quenching enzymes

Quorum quenching refers to the enzymatic or chemical disruption of QS signaling molecules. Enzymatic quorum quenching is mainly mediated by AHL lactonases, AHL acylases, oxidoreductases, and other signal-modifying enzymes (Sikdar & Elias, 2020; D'Aquila et al., 2024). These enzymes can degrade or modify autoinducers, thereby preventing their interaction with cognate receptors and reducing QS-regulated virulence.

AHL lactonases hydrolyze the lactone ring of AHL molecules, whereas AHL acylases cleave the amide bond between the acyl chain and the homoserine lactone ring. Oxidoreductases may modify the acyl side chain and alter signal activity (Sikdar & Elias, 2020). These enzymatic mechanisms can reduce biofilm formation, pigment production, motility, extracellular enzyme secretion, and other QS-regulated phenotypes in Gram-negative bacteria.

The main advantage of quorum-quenching enzymes is their mechanistic specificity. Unlike crude extracts or nanoparticles, enzymes can be directly linked to signal degradation. However, their application faces several limitations, including enzyme instability, delivery barriers, immunogenicity, production cost, and reduced activity under physiological conditions (Sikdar & Elias, 2020; D'Aquila et al., 2024). Immobilization on biomaterials, encapsulation in nanoparticles, incorporation into coatings, and protein engineering may improve their stability and practical applicability.

3.6. Synthetic and semi-synthetic QS inhibitors

Synthetic and semi-synthetic QS inhibitors include signal analogues, receptor antagonists, small molecules, halogenated furanone derivatives, AHL analogues, quinolone analogues, peptide analogues, and hybrid molecules. These compounds are designed to interfere with specific QS components, including LuxR-type receptors, LasR, RhlR, PqsR, AgrC, AgrA, or AI-2-associated pathways (Hetta et al., 2024; Naga et al., 2024).

Synthetic QS inhibitors are valuable because their chemical structures can be optimized for potency, selectivity, stability, and pharmacokinetic properties. Structure–activity relationship studies, molecular docking, molecular dynamics simulations,

medicinal chemistry, and high-throughput screening can support rational design (Hetta et al., 2024; Liu et al., 2024). For example, small molecules targeting QS pathways in *P. aeruginosa* may reduce biofilm formation, pigment production, virulence gene expression, and QS-controlled pathogenic traits (Liu et al., 2024).

Nevertheless, synthetic QS inhibitors also face translational challenges. Compounds identified through reporter strains, docking studies, or simplified in vitro assays may fail in complex biofilm systems, polymicrobial infections, or in vivo models (Naga et al., 2024; Hetta et al., 2024). Therefore, synthetic candidates should be evaluated through growth-independent virulence assays, QS gene expression analysis, signal molecule quantification, cytotoxicity assessment, and infection-relevant validation.

3.7. Combination strategies

Bioactive compounds targeting QS may be used alone, but their greatest therapeutic value may lie in combination strategies. QS inhibitors can weaken biofilm structure, reduce toxin production, impair bacterial coordination, increase antibiotic penetration, and improve immune clearance (Wang et al., 2024; Bhuiyan et al., 2025). In this context, anti-QS agents may act as adjuvants rather than stand-alone antimicrobials.

Combination strategies may involve phytochemicals plus antibiotics, nanoparticles plus antibiotics, postbiotics plus conventional antimicrobials, biosurfactants plus antibiofilm agents, or quorum-quenching enzymes incorporated into biomaterials (Wang et al., 2024; D'Aquila et al., 2024). Such approaches are particularly relevant for biofilm-associated infections, where bacterial tolerance results from matrix protection, slow growth, persister cells, limited drug penetration, and QS-mediated community behavior.

However, combination studies require rigorous experimental design. Synergy should be assessed using checkerboard assays, fractional inhibitory concentration indices, time-kill curves, biofilm inhibition assays, biofilm eradication assays, and dose-response modeling (Wang et al., 2024). Importantly, synergy against bacterial growth does not necessarily indicate synergy against QS-regulated virulence. Therefore, combination studies should evaluate both antimicrobial and antivirulence endpoints.

3.8. Methodological considerations for studying bioactive anti-QS compounds

The study of bioactive compounds targeting QS systems requires strict methodological caution. The first essential step is MIC determination. Anti-QS assays should generally be performed at sub-MIC concentrations to avoid confusing growth inhibition with virulence attenuation (Hetta et al., 2024; Naga et al., 2024). Growth curves should be included to confirm that tested concentrations do not significantly impair bacterial proliferation during the assay period.

Second, multiple virulence phenotypes should be evaluated. A single assay, such as biofilm biomass reduction or violacein inhibition, is not sufficient to establish anti-QS activity (D'Aquila et al., 2024; Bhuiyan et al., 2025). Stronger evidence comes from concordant reductions in several QS-regulated phenotypes, including biofilm formation, motility, pigment production, extracellular enzyme activity, toxin secretion, and siderophore production.

Third, molecular confirmation is necessary. RT-qPCR, transcriptomics, proteomics, metabolomics, reporter assays, or direct signal detection should be used to determine whether the tested compound affects QS-related genes, proteins, metabolites, or signaling molecules (Hetta et al., 2024; Hu et al., 2024). For *P. aeruginosa*, relevant genes may include *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, and *pqsR*. For *S. aureus*, they may include *agrA*, *agrC*, RNAIII, *hla*, and *psm* genes. For *A. baumannii*, *abaI* and *abaR* may be relevant.

Fourth, compound-specific controls are required. For plant extracts, chemical characterization and solvent controls are essential (Papaneophytou, 2026; Alum et al., 2025). For essential oils, emulsifier controls, volatility considerations, and GC-MS profiling are necessary (Touati et al., 2025; Maggio et al., 2025). For postbiotics, medium controls, pH-neutralized controls, and heat- or enzyme-treated fractions may be required (Amobonye et al., 2025; Tomičić et al., 2025). For nanoparticles, ion release controls, precursor controls, coating controls, and physicochemical characterization are indispensable (Hu et al., 2024; Afrasiabi et al., 2024). For peptides, proteolytic stability and cytotoxicity should be assessed (Khan et al., 2025).

Finally, translation requires biologically relevant models. Compounds that show anti-QS activity in planktonic cultures should be tested in biofilm models, multispecies communities, ex vivo infection models, and in vivo systems such as *Galleria*

mellonella, zebrafish, or murine models (Naga et al., 2024; D'Aquila et al., 2024). Without this progression, anti-QS activity remains preliminary and may not reflect true therapeutic potential.

3.9. Synthesis

Bioactive compounds targeting QS systems represent a broad and promising field for anti-virulence therapy. Plant-derived metabolites, essential oils, marine compounds, probiotics, postbiotics, biosurfactants, bacteriocins, antimicrobial peptides, quorum-quenching enzymes, synthetic inhibitors, and nanoparticles can interfere with bacterial communication and reduce QS-regulated virulence factors (Alum et al., 2025; Papaneophytou, 2026; Hu et al., 2024; Hetta et al., 2024).

However, their diversity also creates major methodological challenges. Anti-QS activity should not be claimed solely on the basis of reduced biofilm formation, pigment production, motility, or enzyme activity. Such effects may result from antibacterial activity, metabolic stress, membrane disruption, pH changes, chemical interference with assays, or nonspecific toxicity (Naga et al., 2024; Bhuiyan et al., 2025). A rigorous interpretation requires integrated evidence from sub-MIC testing, growth controls, phenotypic assays, molecular analyses, signal detection, imaging, and biologically relevant models. This methodological rigor is essential for transforming bioactive anti-QS compounds from promising laboratory observations into credible anti-virulence candidates.

A wide variety of natural, microbial, synthetic, and nanostructured compounds have been investigated for their ability to interfere with bacterial communication systems. Because these compounds act through distinct mechanisms and exhibit different methodological constraints, their classification is essential for understanding current anti-QS research. The principal classes of bioactive compounds evaluated as quorum sensing inhibitors are presented in Table 2.

Table 2. Classes of bioactive compounds investigated for anti-QS activity

Class of bioactive compounds	Representative compounds or sources	Main anti-QS mechanisms	QS-regulated phenotypes commonly evaluated	Key methodological considerations	References
Plant-derived phenolics and flavonoids	Quercetin, luteolin, kaempferol, naringenin, apigenin, catechin, gallic acid, caffeic acid, ferulic acid, chlorogenic acid	Receptor antagonism, inhibition of AHL-mediated signaling, suppression of QS-regulated transcription, interference with virulence gene expression	Biofilm formation, pyocyanin production, elastase activity, motility, protease activity, toxin secretion	Should be tested at sub-MIC levels with growth controls, solvent controls, biosensor confirmation, and RT-qPCR validation	Papaneophytou, 2026; Helcman et al., 2025; Hetta et al., 2024
Tannins and polyphenolic extracts	Hydrolysable tannins, condensed tannins, tannin-rich plant extracts	Interference with adhesion, disruption of extracellular polymeric substances, modulation of enzyme activity, inhibition of QS-associated phenotypes	Biofilm biomass, adhesion, extracellular enzyme activity, protease activity, elastase activity	Possible interference with colorimetric and enzymatic assays; assay-interference controls are required	Alum et al., 2025; Papaneophytou, 2026
Alkaloids	Plant-derived alkaloids and alkaloid-rich fractions	Receptor antagonism, enzyme inhibition, modulation of bacterial regulatory pathways, suppression of virulence gene expression	Pigment production, biofilm formation, motility, virulence gene expression	Anti-QS interpretation should be supported by cytotoxicity testing and confirmation that effects are not due to direct antibacterial activity	Alum et al., 2025
Terpenoids and essential oil constituents	Thymol, carvacrol, eugenol, cinnamaldehyde, limonene, linalool, menthol, geraniol	Disruption of QS-regulated phenotypes, modulation of membrane-associated signaling, inhibition of biofilm and virulence-factor production	Adhesion, motility, biofilm formation, pigment production, toxin secretion, extracellular enzyme release	Because many terpenoids are hydrophobic and membrane-active, membrane integrity assays, leakage assays, and growth controls are essential	Touati et al., 2025; Maggio et al., 2025; Srinivasan et al., 2025

Essential oils and complex plant extracts	Volatile oil mixtures rich in terpenes, terpenoids, and phenylpropanoids	Multitarget inhibition of QS-regulated phenotypes, possible modulation of QS gene expression, biofilm architecture disruption	Motility, pigment production, extracellular enzymes, biofilm biomass, biofilm architecture, QS gene expression	Chemical characterization by GC-MS, HPLC, or LC-MS is required because composition varies with plant origin, extraction method, and storage	Touati et al., 2025; Maggio et al., 2025; Srinivasan et al., 2025; Wang et al., 2025
Marine natural products	Halogenated furanones, cyclic peptides, diketopiperazines, brominated metabolites, marine phenolics, polysaccharides, fungal secondary metabolites	Destabilization of LuxR-type regulators, receptor competition, inhibition of signal production, suppression of virulence gene expression	AHL-mediated biosensor response, biofilm formation, adhesion, motility, pigment production, virulence gene expression	Require purification, structural elucidation, toxicity assessment, and validation in infection-relevant models	Mazumder et al., 2024; Coolahan et al., 2025; D'Aquila et al., 2024; Sikdar & Elias, 2020
Probiotics	Lactobacillus spp., Bifidobacterium spp., and other beneficial microbial strains	Secretion of antimicrobial metabolites, organic acid production, biosurfactant release, enzymatic signal degradation, competition for adhesion sites	Biofilm formation, adhesion, co-aggregation, motility, virulence gene expression	Effects of live cells must be distinguished from heat-killed cells, cell-free supernatants, neutralized supernatants, and purified metabolites	Maftei et al., 2026; Erdinc et al., 2025
Postbiotics	Cell-free supernatants, inactivated microbial preparations, organic acids, peptides, enzymes, exopolysaccharides, biosurfactants	Inhibition of autoinducer accumulation, degradation or neutralization of signals, suppression of QS gene expression, disruption of biofilm matrix	Biofilm formation, adhesion, motility, surface hydrophobicity, virulence gene expression, extracellular enzyme activity	Medium controls, pH-neutralized controls, heat-treatment controls, enzyme-treatment controls, and metabolomic characterization are recommended	Amobonye et al., 2025; Erdinc et al., 2025; Tomičić et al., 2025; Maftei et al., 2026
Biosurfactants	Glycolipids, lipopeptides, rhamnolipid-like molecules, probiotic-derived biosurfactants	Reduction of surface adhesion, interference with biofilm maturation, modification of bacterial surface hydrophobicity, disruption of cell–surface interactions	Adhesion, biofilm biomass, biofilm architecture, motility, cell-surface hydrophobicity	Must be distinguished from direct membrane-disruptive or growth-inhibitory effects using sub-MIC and viability assays	Adnan et al., 2023; Khairunnisa et al., 2024
Bacteriocins and antimicrobial peptides	Ribosomally synthesized bacteriocins, synthetic peptides, peptide-based QS disruptors	Disruption of peptide-mediated QS, interference with receptor activation, inhibition of virulence regulation, membrane-associated effects	Biofilm formation, toxin production, hemolysis, adhesion, QS-regulated gene expression	Because peptides may be antimicrobial, anti-QS activity should be demonstrated at non-lethal concentrations with viability confirmation	Khan et al., 2025; Erdinc et al., 2025
Quorum-quenching enzymes	AHL lactonases, acylases, oxidoreductases, signal-degrading enzymes	Enzymatic degradation or modification of QS signaling molecules, especially AHLs	AHL biosensor response, biofilm formation, motility, pigment production, virulence gene expression	Direct signal-degradation assays should be combined with phenotypic and molecular confirmation	Sikdar & Elias, 2020; D'Aquila et al., 2024
Synthetic and semi-synthetic QS inhibitors	AHL analogues, receptor antagonists, signal-mimicking molecules, designed QS blockers	Inhibition of autoinducer synthesis, receptor antagonism, signal transduction blockade, suppression of QS-regulated transcription	Biosensor inhibition, pyocyanin production, elastase activity, motility, biofilm formation, QS gene expression	Molecular docking should not be used alone; experimental validation with biosensors, sub-MIC testing, and gene-expression analysis is required	D'Aquila et al., 2024; Hetta et al., 2024; Naga et al., 2024

Nanoparticles and nanostructured materials	Silver nanoparticles, zinc oxide nanoparticles, chitosan nanoparticles, lipid-based nanocarriers	Signal interference, oxidative stress modulation, membrane interaction, biofilm matrix destabilization, targeted delivery of anti-QS agents	Biofilm biomass, biofilm viability, EPS production, motility, pigment production, virulence gene expression	Particle characterization, dose standardization, cytotoxicity testing, ion-release controls, and distinction between antibacterial and anti-QS effects are essential	Hu et al., 2024; Gad et al., 2024; Talla et al., 2023
Combination-based anti-QS strategies	Bioactive compound–antibiotic combinations, phytochemical–nanoparticle systems, postbiotic–antibiotic combinations	Synergistic reduction of QS-regulated virulence, biofilm sensitization, attenuation of antimicrobial tolerance	Biofilm biomass, viable biofilm cells, antibiotic susceptibility, virulence-factor production, QS gene expression	Synergy should be assessed using checkerboard assays, FICI, time-kill analysis, biofilm viability assays, and toxicity evaluation	Hetta et al., 2024; Naga et al., 2024; Bhuiyan et al., 2025

4. Experimental Design and General Methodological Considerations

The investigation of bioactive compounds targeting quorum sensing-related virulence factors requires an experimental design that goes beyond conventional antimicrobial screening. In classical antibacterial testing, the central objective is usually to determine whether a compound inhibits bacterial growth or kills bacterial cells. In anti-quorum sensing research, the objective is more specific: to determine whether the compound attenuates communication-regulated virulence without merely reducing bacterial viability. This distinction is essential because quorum sensing is closely connected to bacterial density, growth phase, metabolism, stress adaptation, biofilm development, and environmental sensing (Hetta et al., 2024; Naga et al., 2024). Therefore, a reduction in biofilm formation, pigment production, motility, toxin secretion, or extracellular enzyme activity cannot automatically be interpreted as evidence of direct QS inhibition.

A major difficulty in anti-QS studies is that many virulence-associated phenotypes are not exclusively controlled by quorum sensing. Biofilm formation, for example, is influenced by adhesion, surface properties, extracellular polymeric substances, nutrient availability, oxygen gradients, stress responses, and growth kinetics. Similarly, pigment production or extracellular enzyme activity may be affected by metabolic changes, redox imbalance, nutrient limitation, or compound interference with the assay itself. This explains why anti-QS studies must be designed as progressive investigations in which phenotypic results are supported by growth analysis, appropriate controls, molecular validation, and careful statistical interpretation (Allkja et al., 2021; Redfern et al., 2024; Andersen et al., 2024).

The reliability of anti-QS investigations depends on the coherence of several methodological elements: the selection of bacterial strains, the standardization of culture conditions, the determination of MIC and sub-MIC concentrations, the use of suitable controls, and the interpretation of data through adequate replication and statistical analysis. These parameters are not secondary technical details; they determine whether the observed effect can be interpreted as true virulence attenuation or merely as nonspecific antibacterial stress.

A generalized integrated workflow commonly used for the methodological evaluation of anti-quorum sensing compounds is presented in Figure 4.

4.1 Selection and characterization of bacterial strains

The selection of bacterial strains is one of the first methodological decisions that shapes the interpretation of anti-QS studies. Quorum sensing systems vary considerably between bacterial species and even between strains of the same species. This variability may concern signal production, receptor functionality, virulence expression, biofilm-forming ability, resistance profile, and response to environmental stress. For this reason, the strain used in an anti-QS assay is not simply a biological support for the experiment; it is a determinant of the biological meaning of the results.

4.1.1 Reference strains

Reference strains are widely used because they provide stable, standardized, and well-characterized experimental models. In *Pseudomonas aeruginosa*, strains such as PAO1, PA14, and ATCC 27853 are frequently used to investigate QS-regulated virulence because their signaling systems, virulence phenotypes, and genetic background are well documented (Miranda et al., 2022; Amer et al., 2025). These strains are particularly useful for evaluating pyocyanin production, elastase activity, rhamnolipid production, motility, biofilm formation, and expression of *las*, *rhl*, and *pqs* genes.

Reference strains are also valuable in preliminary screening because they allow reproducibility and comparison across studies. For example, *Chromobacterium violaceum* CV026 is commonly used as an AHL biosensor because violacein production provides a visible indicator of QS-related activity. However, reference strains also have limitations. Long-term laboratory maintenance may modify virulence expression, stress responses, metabolic behavior, and biofilm-forming capacity. Therefore, results obtained exclusively with reference strains should not be overgeneralized, especially when the objective is to evaluate compounds intended for clinically relevant infections.

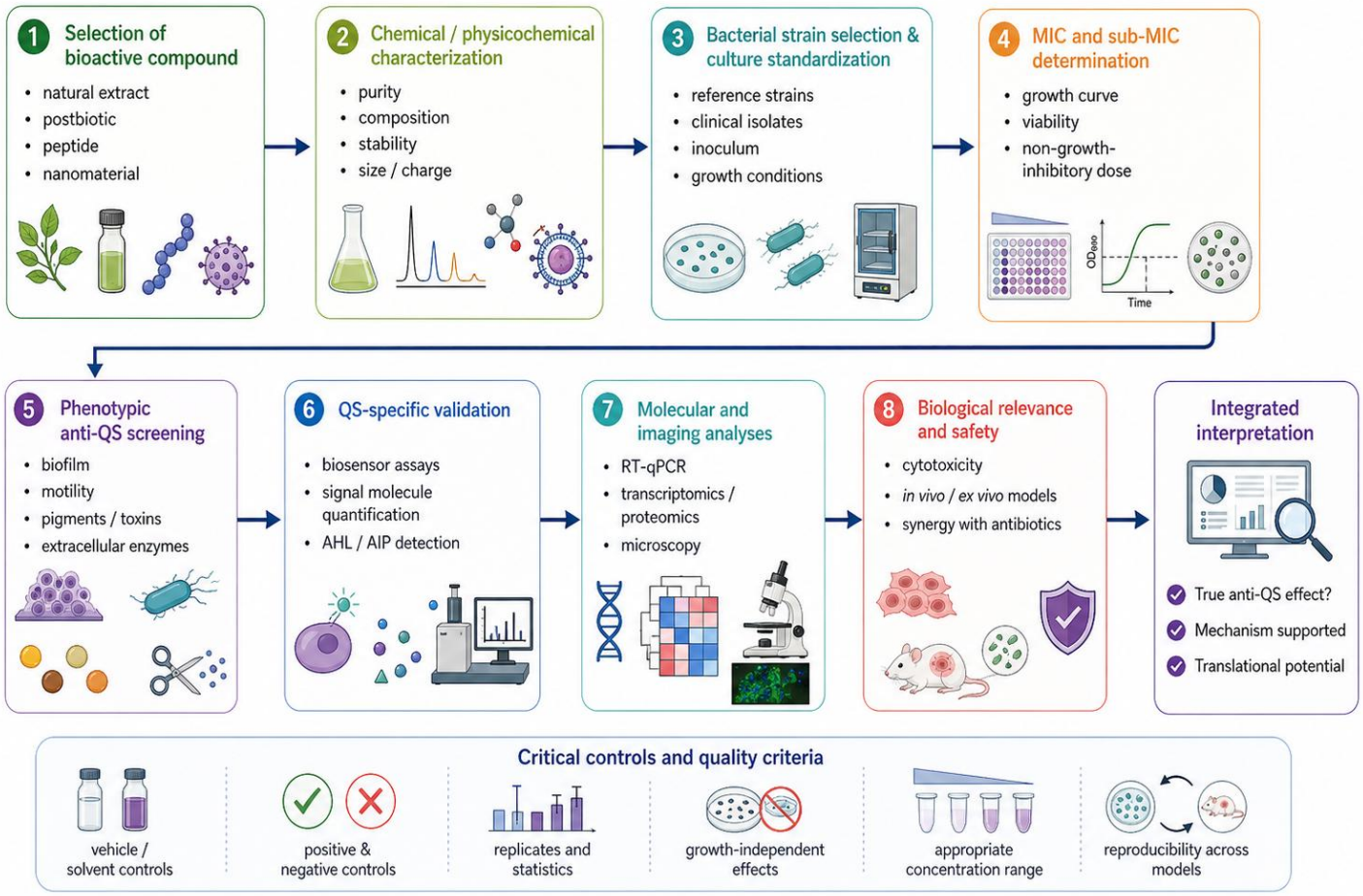

Figure 4. Integrated experimental workflow for anti-QS studies

4.1.2 Clinical isolates

Clinical isolates provide greater biological relevance because they reflect the diversity of pathogens encountered in real infections. Unlike reference strains, clinical isolates have been exposed to host immunity, antimicrobial pressure, nutrient limitation, and polymicrobial competition. As a result, they may display altered QS activity, enhanced biofilm formation, modified virulence expression, and heterogeneous responses to bioactive compounds.

This is particularly important for *P. aeruginosa*. Clinical isolates from chronic airway infections may present mutations or functional alterations in QS-associated regulators such as *lasR*, which can modify virulence expression and reshape the organization of the QS network (Miranda et al., 2022; Zhang et al., 2025; Zhao et al., 2023). Similarly, clinical isolates of *Staphylococcus aureus* may differ in agr activity, toxin production, adhesion properties, biofilm phenotype, and antimicrobial resistance profile (Yamazaki et al., 2024; Rimi et al., 2024). These differences can strongly influence the apparent activity of anti-QS compounds.

The infection source of the isolate should also be considered. Strains recovered from wounds, respiratory secretions, urine, oral biofilms, blood cultures, or medical devices may express different virulence traits because each anatomical niche imposes distinct environmental pressures. Therefore, strain origin, isolation source, resistance phenotype, biofilm-forming capacity, and relevant virulence characteristics should be clearly reported.

4.1.3 Multidrug-resistant strains

Multidrug-resistant strains are particularly relevant in anti-QS research because QS contributes to biofilm formation, persistence, stress tolerance, and reduced susceptibility to antimicrobial therapy. In many clinically important pathogens, including *P. aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and methicillin-resistant *S. aureus*, biofilm-associated growth and QS-regulated virulence contribute to therapeutic failure and chronic infection persistence (Hetta et al., 2024; Bhuiyan et al., 2025).

The inclusion of multidrug-resistant isolates increases the translational relevance of anti-QS studies. However, resistance-associated adaptations may also influence bacterial growth, membrane permeability, metabolic state, and regulatory responses. Therefore, anti-QS effects observed in susceptible strains cannot automatically be extrapolated to resistant isolates. In *A. baumannii*, for example, the *abaI/abaR* QS system has been associated with biofilm development, virulence, and antibiotic susceptibility, making MDR isolates particularly relevant for evaluating compounds that target QS-related pathogenic traits (Sun et al., 2021; Pumirat et al., 2024).

4.2 Culture conditions and bacterial standardization

Culture conditions have a major influence on QS activation because bacterial communication depends on population density and environmental context. Medium composition, pH, iron concentration, oxygen availability, carbon source, osmolarity, temperature, incubation time, and agitation can all modify signal production and virulence factor expression. Therefore, anti-QS studies must describe culture conditions precisely and justify them in relation to the biological question.

4.2.1 Media selection

The choice of culture medium directly affects bacterial growth, metabolism, and virulence expression. Rich media such as LB broth and tryptic soy broth are frequently used because they support rapid growth and are convenient for routine laboratory assays. However, they do not necessarily reproduce infection-relevant environments. In *P. aeruginosa*, QS-regulated phenotypes such as pyocyanin production, elastase activity, rhamnolipid production, and biofilm formation may vary according to nutrient composition, iron availability, and growth conditions (Miranda et al., 2022; Zhang et al., 2025).

The choice of medium may also influence the activity of the tested compound. Plant extracts, essential oils, peptides, postbiotics, and nanoparticles may interact differently depending on pH, ionic strength, proteins, salts, or organic matter. Nanoparticles, in particular, may aggregate, dissolve, adsorb medium components, or undergo surface modifications in biological media, which can affect both antimicrobial and anti-QS outcomes (Hu et al., 2024). Therefore, the medium should not be selected only for convenience; it should be compatible with the target organism, the virulence phenotype measured, and the physicochemical nature of the tested compound.

4.2.2 Growth phase considerations

Growth phase is central to QS research because autoinducer accumulation and QS-regulated gene expression are density-dependent processes. Many virulence factors are expressed preferentially during late exponential or stationary phase, when bacterial density is high enough for signaling molecules to reach an effective threshold. In *P. aeruginosa*, several QS-regulated phenotypes, including pyocyanin production and elastase activity, are strongly influenced by growth phase and incubation duration (Amer et al., 2025).

The impact of growth phase is especially important when studying sub-MIC effects. A compound may appear to reduce virulence at one time point but produce a different effect at another stage of growth. Recent work on *P. aeruginosa* showed that sub-inhibitory antibiotic exposure can modulate QS-related virulence in a growth-phase-dependent manner, illustrating why fixed time-point analysis may be insufficient for interpreting anti-QS effects (Amer et al., 2025). Therefore, studies should define the culture age, incubation duration, and physiological state of bacteria before virulence assays are performed.

4.2.3 Inoculum standardization

Because QS depends on bacterial population density, inoculum standardization is essential. Variations in initial bacterial concentration can alter signal accumulation kinetics, biofilm initiation, virulence expression, and growth behavior. In antimicrobial susceptibility testing, standardized inoculum preparation is a foundational requirement for reproducible MIC determination (Clinical and Laboratory Standards Institute, 2024; European Committee on Antimicrobial Susceptibility Testing, 2024).

Optical density and McFarland standards are commonly used for inoculum adjustment, but turbidity does not always accurately reflect viable cell number. Bacterial aggregation, pigmentation, clumping, biofilm fragments, and differences in cell size may affect optical readings. Therefore, optical density should ideally be correlated with viable counts for each bacterial species and experimental condition. This is particularly important in biofilm assays, where the initial number of adherent cells can strongly influence final biomass and architecture (Allkja et al., 2021; Andersen et al., 2024).

4.3 Determination of MIC and sub-MIC concentrations

Determining MIC is a necessary preliminary step in anti-QS research because virulence assays must be performed at concentrations that do not significantly inhibit bacterial growth. If the tested concentration suppresses growth, decreases viability, or severely alters metabolism, the observed reduction in virulence factors cannot be confidently attributed to QS inhibition.

4.3.1 Broth microdilution methods

Broth microdilution is one of the most widely used methods for MIC determination. It is recommended in standardized antimicrobial susceptibility testing frameworks and allows serial dilution of compounds in microplates under controlled inoculum and incubation conditions (Clinical and Laboratory Standards Institute, 2024; European Committee on Antimicrobial Susceptibility Testing, 2024). Its compatibility with high-throughput screening makes it particularly useful for preliminary evaluation of anti-QS candidates.

However, broth microdilution may be complicated by the physicochemical properties of bioactive compounds. Colored plant extracts may interfere with absorbance readings; essential oils may separate from the aqueous phase; nanoparticles may sediment or scatter light; and postbiotic preparations may modify medium pH. These limitations require adapted controls and, when necessary, complementary methods for assessing bacterial growth and viability (Hu et al., 2024; Redfern et al., 2024).

4.3.2 Agar dilution methods

Agar dilution methods involve incorporating the tested compound into solid medium before bacterial inoculation. This approach is also recognized in antimicrobial susceptibility testing standards and may be useful when liquid turbidity measurements are unreliable (Clinical and Laboratory Standards Institute, 2024). Agar dilution can be helpful for compounds that interfere with broth optical density measurements, although diffusion, volatility, compound stability, and interaction with the agar matrix may influence apparent activity.

For anti-QS studies, agar-based methods may also be relevant for motility assays, pigment observation, and preliminary screening. However, results obtained on solid media should be interpreted cautiously because QS behavior may differ between planktonic, semi-solid, solid-surface, and biofilm-associated growth.

4.3.3 Resazurin-based assays

Resazurin-based assays are frequently used to estimate bacterial metabolic activity. Resazurin is reduced by metabolically active cells into a fluorescent or colorimetric product, allowing indirect assessment of viability. In biofilm research, resazurin has also been used as one of the comparative methods for evaluating viable or metabolically active biofilm cells in microtiter plate systems (Allkja et al., 2021).

This method can be useful when turbidity readings are unreliable, but it also has limitations. Some compounds may chemically reduce resazurin or interfere with fluorescence and absorbance signals. Conversely, compounds that reduce metabolic activity without killing cells may produce apparent viability reductions. Therefore, resazurin-based assays should be interpreted alongside growth curves, CFU counts, or other viability measures when anti-QS activity is being assessed.

4.3.4 Importance of sub-inhibitory concentrations in anti-QS studies

The use of sub-inhibitory concentrations is central to anti-QS research. The aim is to evaluate whether a compound reduces virulence factor expression without directly suppressing bacterial growth. Sub-MIC concentrations are often selected as fractions of the MIC, such as MIC/2, MIC/4, MIC/8, or MIC/16. However, sub-MIC does not automatically mean physiologically neutral. Even below the MIC, a compound may alter growth kinetics, metabolism, membrane integrity, oxidative balance, or stress responses.

This point is particularly important because sub-inhibitory concentrations can themselves modulate QS and virulence in complex ways. In *P. aeruginosa*, sub-MIC exposure to antibiotics has been shown to affect QS-related virulence factors and QS gene expression depending on growth phase (Amer et al., 2025). Earlier work also showed that sub-inhibitory antibiotic exposure can affect QS-dependent virulence factors and biofilm formation in *P. aeruginosa* (Aleanizy et al., 2021). Therefore, anti-QS studies should not rely only on MIC fractions; they should include growth curves, viability confirmation, and dose-response evaluation.

4.4 Controls and experimental reproducibility

Controls determine whether the observed effect can be interpreted as anti-QS activity or as an artifact of growth inhibition, solvent toxicity, assay interference, or compound instability. Because anti-QS studies often involve complex natural products, nanoparticles, postbiotics, essential oils, and colored extracts, control design must be adapted to the tested compound.

The principal phenotypic and molecular methodologies currently used to evaluate quorum sensing-regulated virulence factors are summarized in Figure 5.

4.4.1 Negative controls

Negative controls consist of untreated bacterial cultures maintained under identical experimental conditions. They establish the baseline level of growth, biofilm formation, motility, pigment production, enzyme activity, or reporter signal. Negative controls are essential because QS-regulated phenotypes can fluctuate naturally according to inoculum density, growth phase, medium composition, incubation time, and environmental stress.

In biofilm assays, untreated controls are especially important because biomass formation may vary significantly between experiments. Interlaboratory work on microtiter plate biofilm quantification has shown that reproducibility depends strongly on experimental design, assay selection, and statistical treatment (Allkja et al., 2021). Therefore, untreated controls should be included in every independent experiment rather than assumed from previous runs.

4.4.2 Positive controls

Positive controls are necessary to confirm that the assay can detect QS inhibition or virulence attenuation. Known QS inhibitors, quorum-quenching enzymes, or previously validated anti-QS compounds can be used depending on the organism and assay. Positive controls are particularly useful in biosensor assays, reporter systems, and phenotypic screens because they establish the responsiveness of the method (Hetta et al., 2024; D'Aquila et al., 2024).

However, the choice of positive control should match the QS system under investigation. A compound known to interfere with AHL-mediated signaling in Gram-negative bacteria may not be appropriate for peptide-mediated QS in Gram-positive bacteria. Similarly, an antibiofilm positive control does not necessarily validate a QS-specific assay unless its mechanism is known.

4.4.3 Vehicle controls

Vehicle controls are indispensable when solvents, emulsifiers, dispersants, or stabilizers are used. DMSO, ethanol, methanol, acetone, Tween 80, and similar agents can influence bacterial membranes, adhesion, motility, biofilm formation, and possibly QS-related phenotypes. Therefore, the same vehicle concentration used in treated groups must be included in control groups.

Vehicle controls are especially important for hydrophobic compounds such as essential oils, terpenoids, flavonoids, and some synthetic QS inhibitors. They are also important for nanoparticles, where stabilizers, reducing agents, or coatings may contribute to biological activity. Without vehicle controls, it is impossible to determine whether the observed effect is caused by the tested compound or by the formulation system.

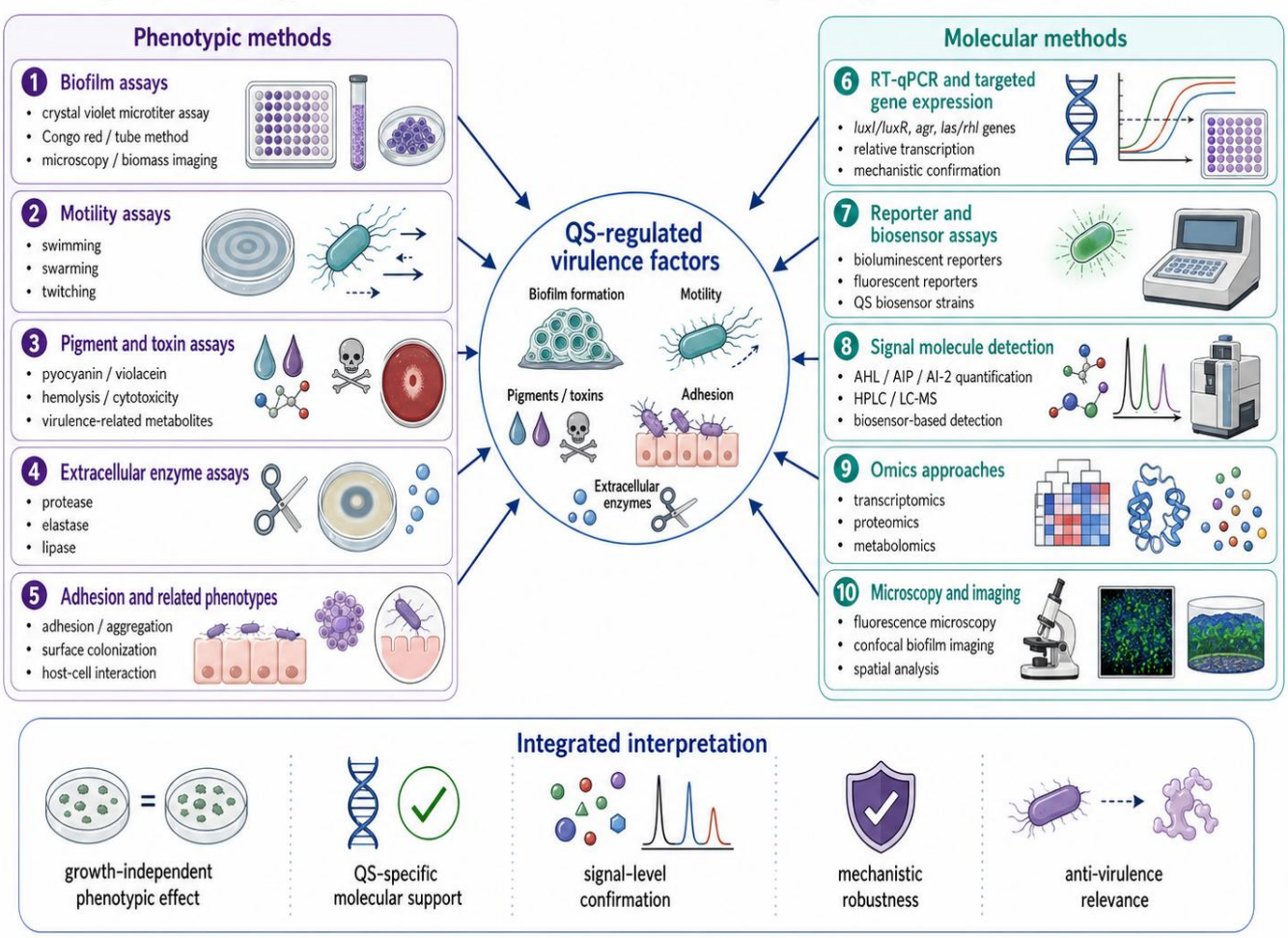

Figure 5. Phenotypic and molecular methods for evaluating QS-regulated virulence factors

4.4.4 Biological and technical replicates

Reproducibility is a major concern in virulence and biofilm assays because these phenotypes are intrinsically variable. Technical replicates measure variability within the same experiment, whereas biological replicates represent independent experiments performed with independently prepared cultures, often on different days. Both levels of replication are necessary.

Microtiter plate biofilm assays illustrate this issue clearly. Although they are widely used, their reproducibility depends on assay design, plate layout, incubation conditions, washing procedure, staining protocol, and data normalization (Allkja et al., 2021; Andersen et al., 2024). In addition, crystal violet assays measure total attached biomass rather than viability or architecture, which means that complementary assays may be required for robust interpretation (Redfern et al., 2024).

4.5 Statistical analysis and data interpretation

Statistical analysis should be planned before experiments are performed. Anti-QS assays often generate variable data because virulence expression depends on multiple biological and environmental parameters. The number of biological replicates, technical replicates, normalization strategy, statistical test, significance threshold, and variability measure should be clearly reported.

Data interpretation must remain biologically cautious. Reporting only percentage inhibition is insufficient if the data are not supported by growth controls, viability measurements, and appropriate statistical analysis. A decrease in crystal violet staining, for example, may indicate reduced biofilm biomass, but it may also reflect impaired growth, altered adhesion, enhanced detachment, or interference with staining. Recent analyses of crystal violet microtiter plate assays emphasize that biofilm development and dispersal dynamics should be considered rather than relying only on a single endpoint measurement (Andersen et al., 2024). Similarly, an apparent reduction in metabolic activity measured by resazurin does not necessarily indicate cell death; it may reflect reduced respiration, altered metabolism, or chemical interference with the dye. Therefore,

statistical significance should not be confused with mechanistic certainty. Reliable conclusions emerge when statistical evidence is integrated with biological controls and mechanistic validation.

Overall, experimental design in anti-QS research must be guided by one principle: virulence attenuation must be distinguished from growth inhibition, physiological stress, and assay interference. A robust study should move progressively from MIC determination to sub-MIC validation, from phenotypic assays to molecular confirmation, and from simple models to biologically relevant systems. This integrated approach is necessary because quorum sensing is not an isolated pathway but a regulatory network embedded in bacterial physiology, ecology, and pathogenesis.

5. Phenotypic Methods for Evaluating Quorum Sensing-Related Virulence Factors

Phenotypic methods remain central to the investigation of quorum sensing (QS)-related virulence because they provide a functional readout of how bacterial populations behave under defined experimental conditions. In anti-QS studies, the objective is not merely to determine whether a compound inhibits bacterial growth, but to establish whether it interferes with collective bacterial traits such as biofilm formation, motility, pigment production, extracellular enzyme secretion, exopolysaccharide synthesis, adhesion, invasion, toxin release, and siderophore production. These phenotypes are particularly relevant because many of them are regulated either directly or indirectly by QS circuits and contribute to bacterial persistence, host damage, immune evasion, and tolerance to antimicrobial treatment (Qin et al., 2022; Filipić et al., 2024; Rodríguez-Urretavizcaya et al., 2024).

A rigorous phenotypic evaluation of bioactive compounds must therefore distinguish between three different effects: antibacterial activity, antibiofilm activity, and antivirulence or anti-QS activity. A reduction in virulence factor production at bactericidal or bacteriostatic concentrations cannot be automatically interpreted as QS inhibition, because the observed effect may simply result from reduced bacterial biomass or metabolic suppression. For this reason, phenotypic assays are generally performed at sub-inhibitory concentrations, preferably after MIC determination and growth-curve verification, so that changes in virulence expression can be interpreted independently from major growth inhibition (Haney et al., 2018; Filipić et al., 2024). Recent anti-virulence studies increasingly combine several phenotypic assays in parallel, rather than relying on a single endpoint, because QS-regulated traits are interconnected but not equivalent. For example, a compound may inhibit violacein production in *Chromobacterium violaceum* without substantially reducing biofilm formation, or reduce pyocyanin production in *Pseudomonas aeruginosa* while having only a moderate effect on motility or elastase activity (Sadik et al., 2024; Wen et al., 2024).

5.1 Biofilm formation and inhibition assays

Biofilm formation is one of the most widely investigated QS-associated phenotypes because biofilms represent structured bacterial communities embedded in a self-produced matrix and are strongly associated with chronic infection, antimicrobial tolerance, immune escape, and persistence on biotic and abiotic surfaces. In several pathogens, including *P. aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*, QS contributes to biofilm maturation, matrix production, dispersal, metabolic adaptation, and intercellular coordination, although the degree and direction of QS involvement may vary between species, strains, and growth conditions (Azeredo et al., 2017; Qin et al., 2022; Beenken et al., 2025). Consequently, antibiofilm assays are frequently used as primary phenotypic tools to evaluate plant extracts, purified natural products, synthetic molecules, nanoparticles, postbiotics, enzymes, and other bioactive compounds with potential anti-QS activity (Shariati et al., 2024; Omran et al., 2024).

The major advantage of biofilm assays is that they provide a biologically meaningful model of bacterial communal behavior. However, their interpretation requires caution. Biofilm formation is influenced by medium composition, surface chemistry, incubation time, temperature, oxygen availability, inoculum density, nutrient limitation, and hydrodynamic conditions. A compound that appears highly active in a static microplate assay may perform differently under flow conditions or on clinically relevant surfaces such as catheters, wound dressings, epithelial cells, or implant materials (Azeredo et al., 2017; Haney et al., 2018). Therefore, antibiofilm activity should ideally be assessed using complementary methods that measure biomass, viable cells, matrix composition, architecture, and metabolic activity rather than relying on a single colorimetric endpoint.

5.1.1 Crystal violet microplate assay

The crystal violet microplate assay remains the most commonly used method for screening biofilm formation and inhibition because it is inexpensive, simple, scalable, and adaptable to 96-well or 24-well plate formats. In this method, bacterial cells are allowed to adhere and develop biofilms on the well surface, planktonic cells are removed, adherent biomass is stained with

crystal violet, and the bound dye is solubilized before absorbance measurement. The method was popularized through standardized static biofilm protocols and remains particularly useful for comparing treated and untreated groups under identical conditions (Merritt et al., 2005; O'Toole, 2011).

The main strength of the crystal violet assay is its suitability for high-throughput screening of large numbers of compounds, concentrations, strains, or extracts. It allows early identification of candidates that reduce total attached biomass and is therefore particularly useful in preliminary anti-QS or antibiofilm studies. It has been widely applied to investigate compounds that interfere with QS-related biofilm formation in *P. aeruginosa*, *S. aureus*, and other clinically relevant bacteria (Haney et al., 2018; Shariati et al., 2024). Nevertheless, the assay does not distinguish living cells from dead cells, bacterial cells from extracellular matrix, or true biofilm inhibition from reduced attachment caused by surface interference. A compound may also interact with crystal violet, alter dye binding, stain the plate surface, or precipitate during incubation, leading to false-positive or false-negative interpretations (Azeredo et al., 2017; Haney et al., 2018).

For this reason, crystal violet results should be interpreted as a measure of total adherent biomass rather than direct biofilm viability. In anti-QS studies, it is preferable to combine this assay with viable cell counts, metabolic assays, microscopy, or matrix quantification. It is also important to normalize biofilm data to bacterial growth when the tested concentration slightly affects planktonic density. Without this normalization, a reduction in crystal violet staining may be incorrectly interpreted as QS inhibition when it actually reflects lower bacterial proliferation.

5.1.2 Tube method

The tube method is an older but still useful qualitative or semi-quantitative approach for detecting biofilm formation. In this method, bacteria are incubated in glass or plastic tubes, the contents are discarded, and the inner wall is stained to visualize attached biomass. The method has historically been used to detect slime and biofilm production in staphylococci and other bacteria, especially when rapid phenotypic classification is needed (Christensen et al., 1985; Stepanović et al., 2000).

Compared with the microplate assay, the tube method is less suitable for high-throughput screening and produces more subjective results because interpretation often depends on visual scoring. However, it can be useful as a complementary assay when the objective is to confirm whether a compound visibly reduces surface adhesion or ring formation at the air–liquid interface. In anti-QS studies, it may serve as a preliminary or confirmatory test, especially in laboratories with limited equipment. Its limitations include low precision, operator-dependent interpretation, poor sensitivity for weak biofilm producers, and difficulty in generating robust quantitative comparisons across multiple compounds or concentrations.

5.1.3 Congo red agar method

The Congo red agar method is commonly used to detect slime production and extracellular polysaccharide-associated phenotypes, particularly in staphylococci. Colonies producing slime or polysaccharide-rich matrix may display characteristic dark, black, dry, or crystalline morphologies depending on the strain and experimental conditions. The method was initially proposed for detecting slime production in coagulase-negative staphylococci and has since been adapted for several biofilm-related studies (Freeman et al., 1989).

In the context of anti-QS and antivirulence screening, Congo red agar can provide useful information on colony morphology and matrix-associated phenotypes. It is especially valuable when the tested compound is suspected to interfere with extracellular polymeric substance production rather than only bacterial adhesion. However, this method remains largely qualitative and highly dependent on medium composition, sugar concentration, incubation temperature, and strain background. It should therefore not be used alone as proof of antibiofilm or anti-QS activity. Its best role is complementary, helping to detect visible changes in matrix-associated colony morphology that can later be validated by polysaccharide quantification, microscopy, or molecular assays.

5.1.4 Flow-cell biofilm systems

Flow-cell systems provide a more dynamic and physiologically informative model of biofilm development than static microplate assays. In these systems, bacteria form biofilms under continuous or semi-continuous nutrient flow, allowing the observation of adhesion, microcolony formation, maturation, channel development, and dispersal under controlled hydrodynamic conditions. When combined with confocal laser scanning microscopy and image analysis software, flow-cell systems allow spatial quantification of biofilm thickness, biomass, roughness, substratum coverage, and architecture (Heydorn et al., 2000; Azeredo et al., 2017).

The strength of flow-cell models lies in their ability to better reproduce biofilm behavior under conditions closer to those found in medical devices, water systems, host surfaces, and chronic infection environments. They are particularly useful for evaluating compounds that may alter biofilm architecture without necessarily reducing total biomass. For example, a compound may produce a thinner, less structured, or more dispersed biofilm while only moderately changing crystal violet absorbance. Such effects can be biologically important because biofilm structure influences nutrient gradients, antimicrobial penetration, immune recognition, and dispersal. However, flow-cell systems require specialized equipment, microscopy expertise, longer experimental times, and more complex image analysis. They are therefore less suitable for large screening campaigns but highly valuable for mechanistic validation after preliminary microplate assays.

5.1.5 Quantification of viable biofilm cells

Because crystal violet and related biomass assays do not distinguish viable from non-viable biomass, quantification of viable biofilm cells is essential when evaluating compounds with possible antibacterial, antibiofilm, or anti-QS properties. Viable biofilm cells can be quantified by disrupting the biofilm through scraping, vortexing, sonication, or enzymatic treatment, followed by serial dilution and colony-forming unit enumeration. This approach provides direct information on the number of culturable cells remaining within the biofilm after treatment (Azeredo et al., 2017; Haney et al., 2018).

Viable cell quantification is particularly important when a compound reduces biofilm biomass but does not kill embedded bacteria, or conversely when it kills biofilm cells without markedly reducing the matrix. In the first case, the compound may interfere with adhesion, EPS synthesis, or QS-regulated biofilm maturation. In the second case, it may behave primarily as an antimicrobial agent against sessile cells. Combining biomass and viability measurements therefore helps distinguish antibiofilm, bactericidal, and antivirulence mechanisms. This distinction is crucial in anti-QS research, where the desired effect is often attenuation of virulence rather than direct killing.

5.2 Motility assays

Bacterial motility is closely associated with colonization, surface exploration, biofilm initiation, dissemination, and host invasion. In *P. aeruginosa*, motility is particularly important because swimming, swarming, and twitching reflect different cellular structures and regulatory networks. Swimming depends mainly on flagella-mediated movement in semi-solid medium, swarming involves coordinated multicellular movement across viscous surfaces and is influenced by flagella, pili, biosurfactants, and QS-regulated rhamnolipid production, while twitching is mediated by type IV pili and contributes to surface-associated movement and microcolony organization (Köhler et al., 2000; Rashid & Kornberg, 2000; Déziel et al., 2003).

Motility assays are frequently included in anti-QS studies because QS can regulate biosurfactant production, flagellar-associated behavior, pili function, and surface adaptation. However, motility phenotypes are highly sensitive to agar concentration, medium composition, humidity, plate drying time, incubation temperature, inoculation method, and strain background. For this reason, small methodological variations can produce large differences in motility zones, especially in swarming assays (Tremblay & Déziel, 2008). A reduction in motility after treatment may indicate interference with QS-regulated virulence, but it may also reflect altered growth, impaired energy metabolism, changes in surface tension, or direct effects of the compound on flagella, pili, or membrane integrity. Therefore, motility data should be interpreted together with growth controls and other virulence assays.

5.2.1 Swimming motility

Swimming motility is usually assessed using low-percentage semi-solid agar that permits flagella-mediated movement away from the inoculation point. The diameter of bacterial migration is measured after incubation and compared between treated and untreated groups. In *P. aeruginosa*, swimming has been used to evaluate the impact of QS-interfering compounds on flagellar movement and early colonization behavior (Rashid & Kornberg, 2000; Wen et al., 2024).

The method is simple and inexpensive, but its interpretation requires careful control of agar concentration and inoculum depth. If the agar is too firm, swimming may be underestimated; if too soft, passive diffusion or spreading may complicate interpretation. In anti-QS studies, swimming inhibition should be considered meaningful only when the compound does not substantially inhibit bacterial growth at the tested concentration.

5.2.2 Swarming motility

Swarming motility reflects coordinated surface translocation and is often more closely linked to QS-regulated group behavior than swimming. In *P. aeruginosa*, swarming depends on flagella, type IV pili, and rhamnolipid biosurfactants, whose production is controlled in part by QS systems. The connection between QS, rhamnolipid production, and swarming makes this assay particularly relevant for evaluating compounds that may interfere with collective surface behavior (Köhler et al., 2000; Déziel et al., 2003).

Swarming assays are informative but notoriously difficult to reproduce. Plate moisture, agar thickness, medium composition, drying time, and incubation atmosphere strongly influence the swarming pattern. Tremblay and Déziel emphasized the importance of standardizing swarming assay conditions to improve reproducibility in *P. aeruginosa* studies (Tremblay & Déziel, 2008). Recent anti-QS studies often include swarming inhibition as part of a broader phenotypic panel, alongside pyocyanin, biofilm, elastase, and violacein assays, rather than treating it as an isolated endpoint (Sadik et al., 2024; Wen et al., 2024).

5.2.3 Twitching motility

Twitching motility is mediated by type IV pili and enables bacteria to move along solid surfaces. In *P. aeruginosa*, twitching contributes to surface colonization, biofilm development, host interaction, and microcolony formation. Twitching assays are usually performed by inoculating bacteria at the interface between agar and the Petri dish surface, followed by staining of the migration zone after incubation.

Although twitching is not always directly controlled by QS in the same manner as pigment or elastase production, it is functionally connected to surface-associated virulence and biofilm development. Therefore, compounds that reduce twitching may affect bacterial colonization capacity, pili function, surface sensing, or regulatory pathways linked to virulence. As with swimming and swarming assays, interpretation requires growth controls and standardized agar conditions.

5.3 Pigment production assays

Pigment production is one of the most useful phenotypic readouts in QS research because several bacterial pigments are directly or indirectly regulated by QS systems and can be quantified spectrophotometrically. Pigment assays are especially valuable because they often provide a clear and measurable signal without requiring complex equipment. However, pigment production is also influenced by medium composition, oxygen availability, iron concentration, incubation time, and bacterial growth phase, which must be carefully standardized.

5.3.1 Pyocyanin quantification

Pyocyanin is a blue-green phenazine pigment produced by *P. aeruginosa* and is one of the best-known QS-regulated virulence factors. It contributes to oxidative stress, epithelial injury, immune modulation, redox imbalance, and competition with other microorganisms. Pyocyanin biosynthesis is linked to phenazine metabolism and regulated through interconnected QS networks, including Las, Rhl, and PQS systems (Essar et al., 1990; Price-Whelan et al., 2006; Qin et al., 2022).

Pyocyanin quantification is commonly performed by extracting the pigment from culture supernatants using organic solvents, followed by acidification and absorbance measurement. The method provides a useful readout of QS-regulated secondary metabolism and has been widely used to evaluate plant-derived compounds, synthetic inhibitors, nanoparticles, and other antivirulence agents targeting *P. aeruginosa* (Rodríguez-Urretavizcaya et al., 2024; Wen et al., 2024). A decrease in pyocyanin production at sub-MIC concentrations may suggest interference with QS-regulated virulence, especially when bacterial growth remains unaffected. However, pyocyanin reduction alone is insufficient to prove QS inhibition because the compound may affect phenazine biosynthesis, redox metabolism, iron homeostasis, or general cellular physiology. Therefore, pyocyanin assays should be combined with gene expression analysis, reporter strains, or other phenotypic QS endpoints.

5.3.2 Violacein inhibition assays

Violacein production by *Chromobacterium violaceum* is one of the most widely used biosensor-based phenotypic systems for detecting QS inhibition. Violacein is a purple pigment regulated by AHL-mediated QS, and its inhibition in biosensor strains provides a convenient screening model for compounds with potential QS-interfering activity. The use of *C. violaceum* as a QS reporter system has been foundational in the study of AHL detection and inhibition (McClean et al., 1997).

Quantitative violacein extraction allows more reliable measurement than visual observation alone. Blosser and Gray developed an extraction-based quantitative assay that improved the ability to measure violacein production and evaluate AHL-related activity in *C. violaceum* systems (Blosser & Gray, 2000). In current anti-QS research, violacein inhibition is frequently used as an initial screening assay before moving to pathogen-specific virulence assays in *P. aeruginosa*, *S. aureus*, or other clinically relevant organisms. For example, recent studies have used violacein inhibition in combination with *P. aeruginosa* assays for biofilm, pyocyanin, motility, and protease production to strengthen the interpretation of anti-QS activity (Sadik et al., 2024).

Nevertheless, violacein inhibition must be interpreted carefully. A compound may reduce violacein because it interferes with QS signaling, but also because it inhibits growth, alters pigment biosynthesis, affects membrane permeability, or chemically interacts with the pigment. Thus, violacein assays require parallel growth measurement and appropriate solvent controls. They are best considered as screening tools rather than definitive proof of anti-QS activity.

5.3.3 Other QS-regulated pigments

Other bacterial pigments may also serve as phenotypic indicators of virulence regulation. In addition to pyocyanin and violacein, phenazine derivatives, prodigiosin-like pigments, staphyloxanthin, and melanin-like pigments may reflect stress adaptation, oxidative protection, immune evasion, or secondary metabolism. However, the degree to which these pigments are directly QS-regulated varies among species. Therefore, when such pigments are used in anti-QS studies, the regulatory relationship must be clearly justified for the organism under investigation.

Pigment assays are attractive because they are simple and visually interpretable, but they should not be overinterpreted. A strong methodological approach requires linking pigment inhibition to bacterial growth data, QS-regulated gene expression, and additional virulence phenotypes. In this sense, pigment production assays are most powerful when integrated into a multi-endpoint antivirulence evaluation strategy.

5.4 Protease and extracellular enzyme activity assays

Extracellular enzymes are major contributors to bacterial virulence because they degrade host tissues, disrupt immune defenses, promote nutrient acquisition, and facilitate dissemination. In several pathogens, especially *P. aeruginosa*, extracellular proteases, elastases, lipases, DNases, and phospholipases are connected to QS-regulated virulence networks. Their measurement is therefore highly relevant in studies evaluating bioactive compounds as potential anti-QS or antivirulence agents (Qin et al., 2022; Rodríguez-Urretavizcaya et al., 2024).

The major advantage of enzyme activity assays is that they provide functional information on secreted virulence factors. However, enzyme assays require careful normalization because reduced activity may result from lower enzyme expression, impaired secretion, direct enzyme inhibition, protein precipitation, or reduced bacterial growth. Therefore, extracellular enzyme activity should ideally be measured in cell-free supernatants collected from cultures grown under standardized conditions and normalized to bacterial density or protein content when appropriate.

5.4.1 Elastase activity

Elastase is one of the most studied extracellular virulence factors of *P. aeruginosa*. The LasB elastase degrades elastin, immunoglobulins, complement components, cytokines, and host structural proteins, contributing to tissue invasion and immune damage. Elastase production is strongly associated with QS regulation, particularly through the Las system, making it a relevant phenotypic marker in anti-QS studies (Ohman et al., 1980; Rust et al., 1994; Qin et al., 2022).

Elastase activity is commonly evaluated using elastin-Congo red or related substrate-based assays, in which degradation of the elastin substrate releases measurable dye into the supernatant. Rust and colleagues described elastase assays that have become widely used for measuring extracellular elastolytic activity in *P. aeruginosa* research (Rust et al., 1994). Reduction of elastase activity at sub-MIC concentrations is often interpreted as evidence of antivirulence activity, especially when accompanied by reduced pyocyanin, protease, biofilm, or motility phenotypes (Wen et al., 2024). However, direct inhibition of elastase enzyme activity by the compound must be distinguished from reduced elastase production. This can be addressed by testing whether the compound inhibits purified enzyme activity or by measuring elastase gene expression.

5.4.2 Gelatinase production

Gelatinase assays are used to evaluate proteolytic degradation of gelatin, usually through agar plate methods or liquid substrate-based assays. In gelatin agar, bacterial protease activity is detected by zones of hydrolysis after incubation and development.

Gelatinase production is relevant in pathogens such as *Enterococcus faecalis*, *Serratia marcescens*, *P. aeruginosa*, and other opportunistic bacteria where extracellular proteases contribute to tissue invasion, nutrient acquisition, and biofilm behavior.

Although gelatinase assays are relatively simple, they are often semi-quantitative and should be interpreted with caution. The size of hydrolysis zones depends not only on enzyme production but also on diffusion, growth rate, medium composition, and incubation conditions. In anti-QS studies, gelatinase inhibition should therefore be supported by quantitative protease assays or molecular data when the objective is to claim QS interference.

5.4.3 Lipase activity

Lipases contribute to bacterial virulence by hydrolyzing host lipids, altering membrane integrity, supporting nutrient acquisition, and facilitating tissue colonization. In *P. aeruginosa* and other pathogens, lipase production may be linked to broader virulence regulation and can be influenced by QS-associated pathways. Lipase activity is commonly detected on tributyrin agar, Tween-containing media, or other lipid substrates where hydrolysis produces visible halos or turbidity changes.

Lipase assays are useful for detecting extracellular enzymatic activity, but they are less specific as QS markers than pyocyanin or elastase in *P. aeruginosa*. Therefore, their value is strongest when they are included as part of a broader extracellular enzyme profile. A bioactive compound that simultaneously reduces lipase, protease, elastase, biofilm formation, and pigment production at sub-MIC concentrations provides stronger evidence of antivirulence activity than a compound affecting only one enzyme endpoint.

5.4.4 DNase assays

DNase activity contributes to virulence by degrading extracellular DNA, modulating biofilm matrix composition, facilitating immune evasion, and promoting tissue invasion. DNase assays are usually performed on DNase agar containing DNA and indicator dyes, where enzymatic degradation produces clear zones around colonies. In biofilm-associated infections, extracellular DNA is an important matrix component, and enzymes that degrade DNA may influence biofilm architecture, dispersal, or immune interactions.

In anti-QS studies, DNase assays may be relevant when investigating pathogens in which nuclease production contributes to virulence or biofilm remodeling. However, DNase activity is not universally QS-regulated across bacteria. Therefore, its inclusion should be justified according to the pathogen studied and interpreted alongside other QS-linked virulence factors.

5.4.5 Lecithinase assays

Lecithinase or phospholipase activity reflects the ability of bacteria to hydrolyze phospholipids such as lecithin. This activity is often assessed on egg-yolk agar, where opaque precipitation zones indicate enzymatic degradation. Lecithinase production is relevant for pathogens that damage host membranes, disrupt tissues, or produce phospholipase-associated virulence factors.

Like lipase and DNase assays, lecithinase assays provide useful phenotypic information but are not always direct indicators of QS inhibition. Their inclusion is most justified when the studied bacterium is known to regulate phospholipase production through QS or when the aim is to construct a broad extracellular virulence profile. In such cases, reduction of lecithinase activity by a bioactive compound can support an antivirulence interpretation, particularly when associated with reduced biofilm formation, motility, pigment production, and toxin release.

5.5 Exopolysaccharide quantification methods

Exopolysaccharides are major components of the biofilm matrix and play essential roles in adhesion, structural stability, water retention, antimicrobial tolerance, immune evasion, and long-term persistence. In *P. aeruginosa*, matrix polysaccharides such as Pel, Psl, and alginate contribute to biofilm architecture and chronic infection phenotypes, although their relative importance varies between strains and conditions. Earlier work showed that alginate is not necessarily the dominant extracellular polysaccharide in all *P. aeruginosa* PAO1 and PA14 biofilms, emphasizing the need to avoid oversimplified assumptions about matrix composition (Wozniak et al., 2003).

EPS quantification is often performed using colorimetric carbohydrate assays, particularly the phenol–sulfuric acid method, which measures total sugars in extracted extracellular polymeric material. The classical method of Dubois and colleagues remains widely used for quantifying sugars and related substances in biological samples (Dubois et al., 1956). In anti-QS and antibiofilm studies, EPS reduction may indicate that a compound interferes with matrix synthesis, biofilm maturation, or

extracellular polymer production. However, total carbohydrate assays lack specificity and cannot identify the precise polysaccharide affected. They may also detect medium-derived sugars, extracellular metabolites, or plant extract residues if sample preparation is not rigorous.

For this reason, EPS quantification should be performed using carefully washed biofilm biomass or cell-free matrix extracts, with appropriate compound-only and medium-only controls. When possible, total carbohydrate measurement should be complemented by specific assays for alginate, extracellular DNA, proteins, or microscopy-based matrix visualization. A compound that reduces EPS content without reducing bacterial growth may be considered a strong candidate for antivirulence investigation, but mechanistic confirmation requires additional QS-related evidence.

5.6 Adhesion and invasion assays

Adhesion and invasion assays provide important information on the ability of pathogens to attach to abiotic surfaces, epithelial cells, immune cells, or medical-device materials. These assays are especially relevant because QS-regulated virulence factors often influence early colonization, surface persistence, tissue invasion, and intracellular survival. Anti-adhesion and anti-invasion effects are increasingly considered part of the antivirulence profile of bioactive compounds, particularly when compounds reduce pathogenic behavior without strong bactericidal activity (Filipić et al., 2024; Bonacorsi et al., 2024).

5.6.1 Abiotic surface adhesion assays

Abiotic adhesion assays evaluate the ability of bacteria to attach to surfaces such as polystyrene, glass, stainless steel, silicone, urinary catheter materials, wound dressing materials, and other clinically or industrially relevant substrates. These assays are often performed as early-stage biofilm assays, where bacterial attachment is measured after short incubation periods before mature biofilm development. Crystal violet staining, viable cell counting, microscopy, or fluorescence-based approaches can be used to quantify adhered cells.

The strength of abiotic adhesion assays is that they can reveal whether a bioactive compound interferes with the initial attachment stage rather than only mature biofilm accumulation. This distinction is important because early adhesion may be affected by surface charge, hydrophobicity, fimbriae, pili, flagella, adhesins, EPS production, and QS-mediated surface adaptation. However, surface properties strongly influence results. A compound may appear anti-adhesive on polystyrene but not on silicone or epithelial cells. Therefore, surface selection should reflect the biological or clinical question being investigated.

5.6.2 Cell line infection models

Cell line infection models are used to evaluate bacterial adhesion, invasion, cytotoxicity, intracellular persistence, and host-cell responses. These models are especially useful when the aim is to move beyond abiotic surfaces and assess whether a compound can reduce bacterial virulence in a host-like environment. Commonly used cell lines include epithelial, intestinal, pulmonary, urinary, keratinocyte, macrophage, and corneal epithelial models, depending on the pathogen and infection site.

Invasion assays typically involve infecting host cells with bacteria, removing non-adherent bacteria, killing extracellular bacteria with antibiotics when intracellular survival is studied, and quantifying associated or internalized bacteria by viable counting, microscopy, or fluorescence-based methods. The relevance of these models has increased with growing evidence that some traditionally extracellular pathogens, including *P. aeruginosa*, can enter and persist within host cells under certain conditions (Resko et al., 2024). Therefore, compounds that reduce adhesion, invasion, or intracellular persistence may have important antivirulence potential even when they do not strongly inhibit planktonic growth.

However, cell line models require careful interpretation. Reduced bacterial recovery may reflect cytotoxicity to host cells, altered host-cell viability, compound interference with antibiotic protection assays, or direct antibacterial effects during infection. Therefore, cytotoxicity assays, bacterial growth controls, and host-cell viability measurements are essential. Anti-adhesion or anti-invasion effects should be interpreted as part of a broader host–pathogen interaction profile rather than as isolated proof of QS inhibition.

5.6.3 Catheter-associated biofilm models

Catheter-associated biofilm models provide clinically relevant platforms for evaluating compounds against biofilms formed on medical-device materials. These models are particularly important for pathogens such as *S. aureus*, coagulase-negative staphylococci, *P. aeruginosa*, *E. faecalis*, *Candida* spp., and Gram-negative opportunists associated with device-related

infections. Catheter segments can be incubated with bacterial suspensions in the presence or absence of test compounds, followed by biomass staining, viable cell recovery, microscopy, or matrix analysis.

Compared with standard microplate assays, catheter models better reproduce surface properties, geometry, and material-associated bacterial adhesion. They are therefore more informative for compounds intended to prevent or treat device-associated infections. However, they are less suitable for high-throughput screening and require careful standardization of catheter material, segment size, inoculum density, incubation time, washing steps, and biofilm recovery methods. Ideally, catheter biofilm assays should be used after initial screening has identified promising compounds in simpler systems.

5.7 Hemolysis and toxin production assays

Hemolysis and toxin production assays are important tools for assessing whether bioactive compounds reduce host-damaging virulence traits. In *S. aureus*, the accessory gene regulator agr system controls the expression of several secreted toxins, enzymes, and hemolysins, making hemolysis a relevant phenotypic marker in anti-QS and antivirulence studies. Recent work has further emphasized the role of the agr quorum-sensing system in priming *S. aureus* virulence under stress conditions, supporting the relevance of toxin-related assays in the evaluation of QS-targeting strategies (Podkowik et al., 2024).

Hemolysis assays are commonly performed using blood agar plates or erythrocyte suspension assays. On blood agar, hemolytic activity is assessed by observing zones of red blood cell lysis around colonies, whereas liquid assays allow more quantitative measurement of hemoglobin release. A decrease in hemolytic activity at sub-inhibitory concentrations may suggest that a compound attenuates toxin production or secretion. However, such results must be carefully interpreted because compounds may directly protect erythrocytes, interfere with hemoglobin absorbance, inhibit bacterial growth, or chemically inactivate toxins. Therefore, appropriate controls are required, including erythrocytes exposed to compound alone, bacterial growth controls, and, where possible, toxin-specific assays.

Toxin production assays may also involve ELISA, Western blotting, reporter strains, cytotoxicity assays on host cells, or mass spectrometry-based detection of secreted proteins. In *S. aureus*, toxin-related phenotypes are especially important because biofilm formation and toxin production may be inversely regulated depending on the infection stage and agr activity. This means that a compound reducing hemolysis may not necessarily reduce biofilm formation, and a compound inhibiting biofilm may not automatically suppress toxin release. Therefore, antivirulence evaluation should consider the biological context of infection and the regulatory balance between adhesion, persistence, dispersal, and toxin-mediated damage (Beenken et al., 2025).

5.8 Siderophore production assays

Siderophores are iron-chelating molecules produced by bacteria to acquire iron under limiting conditions. Because iron availability strongly influences bacterial metabolism, host colonization, oxidative stress responses, biofilm development, and virulence, siderophore production is an important phenotypic trait in pathogenic bacteria. In *P. aeruginosa*, siderophores such as pyoverdine and pyochelin contribute to iron acquisition and are linked to virulence regulation, host damage, and microbial competition. Although siderophore production is not always classified as a direct QS phenotype in all organisms, it frequently intersects with QS-regulated networks, biofilm behavior, and virulence expression (Qin et al., 2022; Rodríguez-Urretavizcaya et al., 2024).

The chrome azurol S assay is the most widely used universal method for detecting siderophore production. Schwyn and Neilands developed the CAS assay as a chemical method for detecting and determining siderophores based on the removal of iron from the blue CAS–iron complex, producing a color change that can be measured qualitatively on agar or quantitatively in liquid format (Schwyn & Neilands, 1987). Payne later described approaches for detection, isolation, and characterization of siderophores, establishing important methodological principles for siderophore studies (Payne, 1994).

CAS agar plate assays are useful for screening siderophore production by observing orange or yellow halos around colonies. Modified CAS agar methods have also been proposed to improve visualization and adapt the assay to different organisms and experimental variables (Machuca & Milagres, 2003). In anti-QS or antivirulence studies, a reduction in siderophore halo size or CAS liquid activity after treatment may indicate interference with iron acquisition or siderophore secretion. However, siderophore assays are sensitive to medium iron content, pH, incubation time, bacterial growth, and compound color. Some plant extracts, nanoparticles, and metal-binding compounds may directly chelate iron or interact with CAS reagents, producing misleading results. Therefore, compound-only controls, iron-replete and iron-limited conditions, and growth normalization are essential.

Siderophore inhibition can be biologically meaningful because interference with iron acquisition may weaken bacterial fitness during infection without necessarily killing the organism directly. However, siderophore reduction should not automatically be interpreted as QS inhibition. It may reflect iron chelation by the compound, metabolic stress, impaired secretion, or general growth limitation. The strongest interpretation is obtained when siderophore inhibition occurs together with reduced biofilm formation, pigment production, extracellular enzyme activity, and QS-regulated gene expression at concentrations that do not significantly affect growth.

Integrated interpretation of phenotypic anti-QS assays

Phenotypic assays are indispensable for evaluating the biological effects of bioactive compounds on QS-related virulence, but no single method is sufficient to establish anti-QS activity. Crystal violet staining may show reduced biofilm biomass, but it does not identify whether the compound affects living cells, matrix production, adhesion, or staining chemistry. Motility assays may reveal impaired swimming, swarming, or twitching, but these phenotypes are highly sensitive to medium and surface conditions. Pigment assays such as pyocyanin and violacein quantification are highly informative, but pigment inhibition may result from pathway-specific metabolic effects rather than direct QS blockade. Enzyme assays provide functional virulence readouts, but reduced activity may reflect impaired secretion, direct enzyme inhibition, or lower bacterial density. Adhesion, invasion, hemolysis, toxin, and siderophore assays broaden the biological relevance of the study, but they require carefully designed controls to avoid overinterpretation.

A robust methodological strategy should therefore combine complementary phenotypic endpoints. For *P. aeruginosa*, a convincing anti-QS phenotypic profile may include reduced pyocyanin, elastase, protease, swarming motility, and biofilm formation at sub-MIC concentrations, with minimal impact on growth. For *C. violaceum*, violacein inhibition provides a useful QS-screening endpoint, but it should be followed by pathogen-specific assays. For *S. aureus*, biofilm formation, hemolysis, toxin production, and agr-related phenotypes should be interpreted together because QS may promote dispersal and toxin expression while reducing surface-associated persistence under certain conditions. For device-associated pathogens, catheter biofilm models and viable cell quantification provide stronger translational relevance than static microplate assays alone.

The most reliable studies are those that move progressively from simple screening assays to more biologically informative validation models. High-throughput methods such as crystal violet staining, violacein inhibition, and agar-based enzyme assays are useful for initial screening. Quantitative assays such as viable biofilm counts, pyocyanin extraction, elastase activity, EPS quantification, CAS liquid assays, and erythrocyte hemolysis provide stronger functional evidence. Advanced models such as flow-cell biofilms, catheter-associated biofilms, microscopy-based architecture analysis, and cell line infection systems provide deeper mechanistic and translational insight. This layered approach reduces the risk of methodological bias and strengthens the interpretation that a bioactive compound attenuates QS-related virulence rather than simply inhibiting bacterial growth or interfering with assay chemistry.

Therefore, phenotypic methods should be viewed not as isolated tests but as an integrated experimental framework. Their value lies in the convergence of evidence: when multiple QS-related virulence traits are consistently reduced under non-growth-inhibitory conditions, and when appropriate controls exclude assay interference, the antivirulence potential of the tested compound becomes scientifically credible. This integrated phenotypic approach provides the necessary foundation for subsequent molecular confirmation through gene expression analysis, reporter systems, proteomics, metabolomics, and in vivo infection models.

Phenotypic assays constitute the first level of evidence in anti-quorum sensing studies because they allow direct evaluation of QS-regulated virulence traits. However, each assay possesses specific strengths and limitations that must be considered when interpreting anti-QS activity. The most commonly used phenotypic assays are summarized in Table 3.

Table 3. Phenotypic assays used for evaluating QS-regulated virulence factors

Phenotypic assay	Main virulence factor or phenotype evaluated	Typical bacterial models	Principle of the assay	Main anti-QS interpretation	Key methodological precautions	References
Crystal violet microplate biofilm assay	Total attached biofilm biomass	*Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli,*	Biofilms are grown in microplates, stained with crystal violet, washed, solubilized,	Reduction of attached biomass may indicate inhibition of adhesion, biofilm initiation, matrix accumulation,	Does not distinguish live cells from dead cells or matrix; requires growth controls, solvent controls, blank wells,	Merritt et al., 2005; O'Toole, 2011; Azeredo et al., 2017; Haney et al., 2018;

		Klebsiella pneumoniae, Acinetobacter baumannii	and quantified by absorbance	or QS-regulated biofilm maturation	and normalization to planktonic growth	Shariati et al., 2024
Tube biofilm method	Surface-associated biofilm and slime formation	*S. aureus,* coagulase-negative staphylococci, *E. faecalis*, Gram-negative opportunists	Bacteria are incubated in tubes, washed, stained, and visually or semi-quantitatively scored for wall-adherent biomass	Visible reduction of ring formation or adherent biomass may suggest impaired surface attachment or biofilm formation	Low precision and operator-dependent interpretation; best used as a complementary qualitative assay	Christensen et al., 1985; Stepanović et al., 2000
Congo red agar method	Slime production and extracellular polysaccharide-associated colony morphology	*S. aureus*, coagulase-negative staphylococci, biofilm-forming clinical isolates	Colonies are grown on Congo red-containing agar and evaluated for color and texture associated with slime or matrix production	Changes in colony morphology may suggest reduced polysaccharide or matrix-associated phenotype	Highly qualitative; affected by medium composition, sugar concentration, incubation temperature, and strain background	Freeman et al., 1989
Flow-cell biofilm assay	Biofilm architecture, thickness, biomass, maturation, dispersal	*P. aeruginosa, S. aureus,* device-associated pathogens	Biofilms are grown under continuous or semi-continuous flow and analyzed by microscopy and image analysis	Altered biofilm architecture under flow may indicate interference with maturation, spatial organization, or dispersal processes	Requires specialized equipment; less suitable for high-throughput screening; should follow preliminary static assays	Heydorn et al., 2000; Azeredo et al., 2017
Viable biofilm cell counting	Number of culturable cells within biofilm	Broadly applicable to bacterial biofilms	Biofilms are disrupted by scraping, vortexing, sonication, or enzymatic treatment, followed by serial dilution and CFU enumeration	Helps distinguish antibiofilm effects from bactericidal activity against sessile cells	Must be combined with biomass assays to differentiate matrix reduction from killing; recovery method must be standardized	Azeredo et al., 2017; Haney et al., 2018
Swimming motility assay	Flagella-mediated movement in semi-solid medium	*P. aeruginosa, E. coli, Salmonella enterica,* Vibrio spp.	Bacteria are inoculated into low-percentage agar and migration diameter is measured	Reduced swimming may indicate impaired flagellar motility, colonization ability, or QS-associated surface behavior	Sensitive to agar concentration, inoculation depth, medium composition, and growth inhibition	Rashid & Kornberg, 2000; Wen et al., 2024
Swarming motility assay	Coordinated multicellular surface movement	*P. aeruginosa, Serratia marcescens,* Proteus spp.	Bacteria are inoculated on semi-solid agar surface and radial surface migration is measured	Reduced swarming may suggest disruption of collective behavior, rhamnolipid production, biosurfactant activity, or QS-regulated surface adaptation	Highly sensitive to plate moisture, agar thickness, drying time, temperature, and medium composition	Köhler et al., 2000; Déziel et al., 2003; Tremblay & Déziel, 2008; Sadik et al., 2024; Wen et al., 2024
Twitching motility assay	Type IV pili-mediated surface movement	*P. aeruginosa* and other piliated bacteria	Bacteria are inoculated at the agar–surface interface; migration zone is stained and measured	Reduced twitching may indicate impaired surface colonization, pili function, microcolony organization, or virulence-associated surface behavior	Not always directly QS-controlled; requires standardized agar conditions and growth controls	Rashid & Kornberg, 2000; Wen et al., 2024
Pyocyanin quantification	QS-regulated phenazine pigment production	*P. aeruginosa*	Pyocyanin is extracted from culture supernatants using organic solvent and quantified spectrophotometrically after acidification	Reduced pyocyanin at sub-MIC concentrations may indicate attenuation of Las/Rhl/PQS-associated virulence regulation	Must exclude growth inhibition, redox interference, altered phenazine metabolism, and compound color interference	Essar et al., 1990; Price-Whelan et al., 2006; Qin et al., 2022; Rodríguez-Urretavizcaya et al., 2024; Wen et al., 2024
Violacein inhibition assay	AHL-regulated pigment production	*Chromobacterium violaceum* wild-type or biosensor strains	Violacein production is visually or quantitatively measured after exposure to test compounds	Inhibition of violacein suggests possible interference with AHL-mediated QS signaling	Screening assay only; requires growth measurement, solvent controls, and confirmation in pathogenic models	McClean et al., 1997; Blosser & Gray, 2000; Sadik et al., 2024

Elastase activity assay	LasB elastase production and extracellular proteolytic virulence	*P. aeruginosa*	Elastin-Congo red or related substrates are incubated with cell-free supernatants and released dye is quantified	Reduced elastase activity may indicate attenuation of Las-regulated extracellular virulence	Must distinguish reduced enzyme production from direct enzyme inhibition; should be supported by gene expression or purified-enzyme controls	Ohman et al., 1980; Rust et al., 1994; Qin et al., 2022; Wen et al., 2024
General protease / gelatinase assays	Extracellular protease activity and tissue-degrading capacity	*P. aeruginosa, S. marcescens, E. faecalis, S. aureus*	Proteolysis is detected on gelatin, skim milk, or protein-containing media, or by liquid substrate assays	Reduced hydrolysis may suggest decreased extracellular enzyme production or secretion	Hydrolysis zones are affected by growth, diffusion, enzyme stability, and medium composition; quantitative confirmation is recommended	Qin et al., 2022; Rodríguez-Urretavizcaya et al., 2024
Lipase assay	Extracellular lipid-degrading enzyme activity	*P. aeruginosa, S. aureus,* opportunistic pathogens	Lipid hydrolysis is detected on tributyrin agar, Tween media, or other lipid substrates	Reduced lipase activity may support broader attenuation of extracellular virulence	Less specific as a QS marker; should be interpreted with other phenotypes such as biofilm, pigment, motility, and protease assays	Qin et al., 2022; Rodríguez-Urretavizcaya et al., 2024
DNase assay	Nuclease activity and extracellular DNA degradation	*S. aureus, P. aeruginosa,* opportunistic pathogens	DNase agar is used to detect DNA degradation through clear zones around colonies	Reduced DNase activity may indicate attenuation of virulence traits linked to tissue invasion or biofilm remodeling	Not universally QS-regulated; inclusion must be justified according to the pathogen studied	Qin et al., 2022; Rodríguez-Urretavizcaya et al., 2024
Lecithinase / phospholipase assay	Phospholipid-degrading activity and membrane-damaging virulence	Clostridium spp., *P. aeruginosa,* other opportunistic pathogens	Egg-yolk agar or phospholipid-containing media reveal opaque precipitation zones	Reduced activity may support an antivirulence profile when phospholipase production is QS-associated	Should not be used alone as evidence of QS inhibition; requires growth and enzyme-interference controls	Qin et al., 2022; Rodríguez-Urretavizcaya et al., 2024
Exopolysaccharide quantification	EPS production and biofilm matrix synthesis	*P. aeruginosa, K. pneumoniae, E. coli, S. aureus*	EPS is extracted from biofilms or cultures and quantified using carbohydrate assays such as phenol–sulfuric acid	Reduced EPS may indicate interference with matrix production, biofilm maturation, or QS-associated polymer synthesis	Total sugar assays lack specificity; medium sugars, plant extracts, and metabolites may interfere	Dubois et al., 1956; Wozniak et al., 2003
Abiotic surface adhesion assay	Initial bacterial attachment to non-living surfaces	Device-associated pathogens, *S. aureus, P. aeruginosa, E. faecalis*	Bacteria are exposed to surfaces such as polystyrene, glass, silicone, stainless steel, or catheter materials and attached cells are quantified	Reduced adhesion may suggest impaired colonization, surface sensing, or early biofilm initiation	Surface chemistry strongly affects results; surface choice should reflect the biological or clinical question	Filipić et al., 2024; Bonacorsi et al., 2024
Cell-line adhesion and invasion assay	Host-cell attachment, invasion, intracellular persistence, cytotoxicity	*P. aeruginosa, E. coli, Salmonella, S. aureus*	Host cells are infected with bacteria; adherent or internalized bacteria are quantified by CFU, microscopy, or fluorescence	Reduced adhesion or invasion may indicate attenuation of host–pathogen interaction and virulence potential	Requires host-cell viability assays, cytotoxicity controls, antibiotic-protection controls, and bacterial growth controls	Filipić et al., 2024; Bonacorsi et al., 2024; Resko et al., 2024
Catheter-associated biofilm model	Biofilm formation on medical-device materials	*S. aureus, P. aeruginosa, E. faecalis,* coagulase-negative staphylococci	Catheter segments are incubated with bacteria and treated compounds; biofilm is quantified by staining, CFU, microscopy, or matrix analysis	Reduced catheter biofilm formation improves translational relevance for device-associated infections	Requires standardization of catheter material, segment size, inoculum, incubation time, washing, and recovery method	Azeredo et al., 2017; Haney et al., 2018; Beenken et al., 2025
Hemolysis assay	Hemolysin production and toxin-mediated red blood cell damage	*S. aureus,* streptococci, hemolytic pathogens	Hemolysis is evaluated on blood agar or by erythrocyte suspension assays measuring hemoglobin release	Reduced hemolysis may indicate attenuation of toxin production, secretion, or Agr-regulated virulence	Compounds may directly protect erythrocytes or interfere with absorbance; erythrocyte-only and compound-only controls are required	Podkowik et al., 2024; Beenken et al., 2025

Toxin production assay	Secreted toxin abundance or activity	*S. aureus, P. aeruginosa, Clostridioides difficile,* pathogenic E. coli	Toxins are measured by ELISA, Western blotting, reporter strains, cytotoxicity assays, or secretome analysis	Reduced toxin production supports an antivirulence effect, especially when QS-regulated toxin networks are known	Must distinguish reduced secretion, direct toxin neutralization, growth inhibition, and host-cell protection	Podkowik et al., 2024; Beenken et al., 2025
Siderophore production assay	Iron-chelating molecule production	*P. aeruginosa, K. pneumoniae, E. coli*, Salmonella spp.	Chrome azurol S agar or liquid CAS assays detect siderophore-mediated iron removal through color change	Reduced siderophore production may indicate impaired iron acquisition and virulence-associated metabolic adaptation	Sensitive to pH, iron content, compound color, and direct iron chelation; compound-only and iron-control conditions are essential	Schwyn & Neilands, 1987; Payne, 1994; Machuca & Milagres, 2003; Qin et al., 2022; Rodríguez-Urretavizcaya et al., 2024

6. Molecular and Omics Approaches

Phenotypic assays are indispensable for demonstrating whether a bioactive compound reduces quorum sensing-related virulence traits, but they cannot, by themselves, establish the molecular basis of the observed effect. A decrease in biofilm formation, pigment production, motility, elastase activity, hemolysis, or siderophore secretion may result from direct interference with QS signaling, but it may also reflect altered bacterial metabolism, stress responses, membrane damage, impaired secretion, reduced growth, or nonspecific inhibition of enzyme activity (Mukherjee & Bassler, 2019; Miranda et al., 2022). Molecular and omics approaches are therefore essential because they allow the investigator to move from a descriptive phenotypic observation to a mechanistic interpretation by examining transcriptional responses, protein abundance, secretome composition, metabolite production, and signal molecule dynamics (Otto et al., 2014; Stark et al., 2019; Pellissier et al., 2021).

In the context of bioactive compounds, the main objective of molecular and omics approaches is not only to confirm that virulence is reduced, but also to identify how this reduction occurs. A compound may downregulate signal synthase genes, inhibit receptor expression, alter transcription of QS-controlled virulence genes, disturb secretion systems, modify extracellular protein profiles, reduce autoinducer concentration, or shift bacterial metabolism toward a less virulent state (Papenfort & Bassler, 2016; Mukherjee & Bassler, 2019; Shariati et al., 2024). Recent studies increasingly combine transcriptomic, proteomic, and metabolomic analyses to understand bacterial biofilms, stress adaptation, virulence regulation, and QS-associated behavior at a systems level rather than through single endpoints only (Wang et al., 2024; Abdelhamid & Yousef, 2024; Zhao et al., 2025). This integrated logic is particularly important for anti-QS research because QS is not an isolated pathway; it is connected to growth phase, nutrient availability, stress response, iron acquisition, secondary metabolism, secretion, biofilm development, and host–pathogen interaction (Mukherjee & Bassler, 2019; Miranda et al., 2022; Juszczuk-Kubiak, 2024).

A strong molecular strategy should therefore be designed after the phenotypic stage. Once sub-inhibitory concentrations have been identified and phenotypic assays have shown reduced virulence without major growth inhibition, molecular analyses can be used to verify whether the compound modulates QS-associated gene expression, protein abundance, secretome composition, metabolite production, or signal molecule levels (Bustin et al., 2009; Taylor et al., 2019; Otto et al., 2014). The most convincing studies are those in which phenotypic attenuation is supported by convergent molecular evidence: reduced virulence phenotype, altered expression of QS genes, decreased production of QS-regulated proteins, and reduced accumulation of relevant signaling molecules or virulence-associated metabolites (Pellissier et al., 2021; Liu et al., 2024; Kristensen et al., 2024).

The major advanced imaging and omics-based methodologies currently used in anti-QS research are summarized in Figure 6.

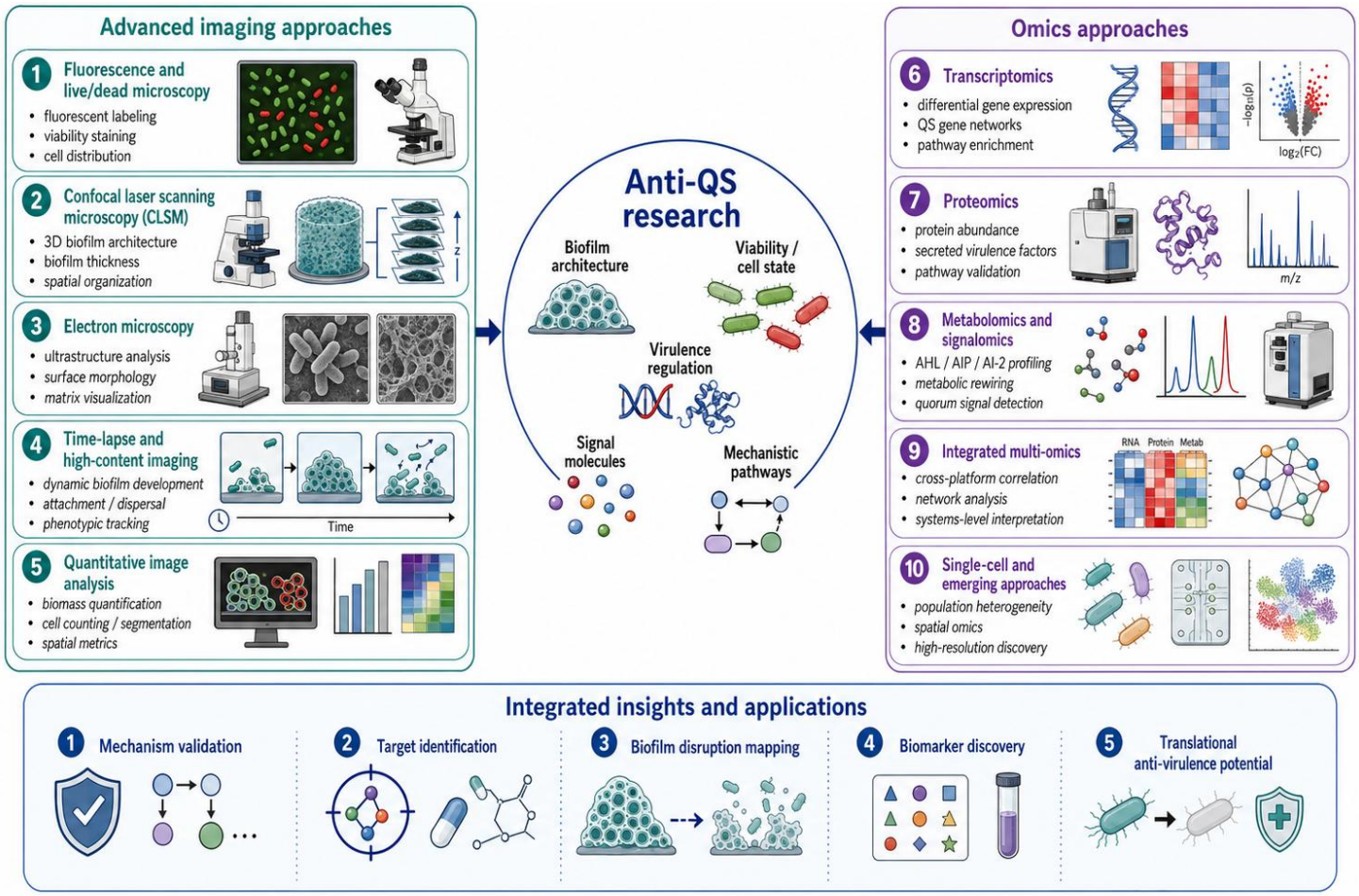

Figure 6. Advanced imaging and omics approaches in anti-QS research

6.1 Gene expression analysis

Gene expression analysis is one of the most direct approaches for evaluating whether bioactive compounds modulate QS-related pathways. It allows comparison between treated and untreated bacteria at the transcriptional level and can reveal whether a compound affects QS regulators, autoinducer synthases, virulence genes, biofilm-associated genes, secretion systems, stress-response genes, and metabolic pathways (Papenfort & Bassler, 2016; Mukherjee & Bassler, 2019; Miranda et al., 2022). In anti-QS studies, gene expression analysis is particularly valuable because many phenotypic traits are regulated by transcriptional networks that respond to cell density, environmental signals, growth phase, and regulatory feedback loops (Papenfort & Bassler, 2016; Mukherjee & Bassler, 2019).

However, gene expression analysis must be carefully designed. The timing of sampling is critical because QS genes are often growth-phase dependent, and differences in optical density or physiological state between treated and untreated cultures may produce apparent transcriptional differences that are not necessarily caused by direct QS inhibition (Bustin et al., 2009; Taylor et al., 2019). For example, in *Pseudomonas aeruginosa*, QS regulation involves interconnected Las, Rhl, and PQS networks whose activity varies according to population density, nutritional conditions, and environmental constraints (Miranda et al., 2022; Kristensen et al., 2024). If samples are collected too early, QS activation may be weak; if collected too late, secondary stress responses may dominate the transcriptional profile. Therefore, cultures should be sampled at a defined optical density or growth phase, and the growth curves of treated and untreated cultures should be compared before RNA extraction (Bustin et al., 2009; Taylor et al., 2019).

6.1.1 RT-qPCR

Reverse transcription quantitative PCR remains the most widely used targeted molecular method in anti-QS studies. Its main advantage is that it allows sensitive and relatively rapid quantification of selected genes known to be involved in QS regulation or QS-controlled virulence, provided that RNA quality, reverse transcription efficiency, primer specificity, amplification

efficiency, and reference gene stability are rigorously controlled (Bustin et al., 2009; Taylor et al., 2019). After RNA extraction, removal of genomic DNA, reverse transcription into cDNA, and quantitative amplification, gene expression is commonly compared between treated and untreated groups using relative quantification approaches such as the $2^{-\Delta\Delta Ct}$ method, with normalization against validated internal reference genes (Livak & Schmittgen, 2001; Vandesompele et al., 2002).

In this context, RT-qPCR is particularly useful for confirming whether phenotypic inhibition is associated with downregulation of genes such as *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, *pqsR*, *aprA*, *lasB*, *rhlA*, *phzA1*, *phzM*, *phzS*, *pelA*, *pslA*, *icaA*, *agrA*, *RNAIII*, or *luxS*, depending on the bacterial species studied and the virulence phenotype being investigated (Miranda et al., 2022; Lai et al., 2024; Juszczuk-Kubiak, 2024). For example, if a compound reduces pyocyanin production in *P. aeruginosa*, RT-qPCR can determine whether this effect is associated with decreased expression of phenazine biosynthetic genes and QS regulators; if a compound reduces elastase activity, expression of *lasB* and upstream Las-system genes may be investigated; and if biofilm formation is reduced, genes involved in matrix synthesis, adhesion, pili, flagella, and polysaccharide production may be selected (Miranda et al., 2022; Liu et al., 2024; Ren et al., 2024).

The strength of RT-qPCR lies in its precision and interpretability when the target genes are well chosen. Recent anti-QS and antivirulence studies have used RT-qPCR to connect phenotypic reductions in virulence with changes in *las*, *rhl*, *pqs*, and downstream virulence gene expression after treatment with bioactive molecules or natural compounds (Liu et al., 2024; Shariati et al., 2024; Kristensen et al., 2024). In *S. aureus*, reduction of hemolysis or toxin production may be linked to changes in *agrA*, *agrC*, *hla*, phenol-soluble modulin genes, or *RNAIII* expression, because the agr system is strongly involved in the regulation of secreted virulence factors and infection-stage adaptation (Lai et al., 2024; Yamazaki et al., 2024).

Despite its usefulness, RT-qPCR remains a targeted method. It only measures genes selected by the investigator and may miss unexpected regulatory effects outside the chosen panel (Conesa et al., 2016; Stark et al., 2019). Another limitation is that transcriptional downregulation does not always correspond to reduced protein abundance or reduced virulence factor activity, because post-transcriptional regulation, protein stability, secretion efficiency, enzyme inhibition, and metabolite availability may all influence the final phenotype (Otto et al., 2014; Solis et al., 2014). Therefore, RT-qPCR should be considered a strong confirmatory tool but not the only molecular evidence. Its value is greatest when combined with phenotypic assays and, when possible, proteomic or metabolomic data (Otto et al., 2014; Pellissier et al., 2021).

6.1.2 Transcriptomic analysis

Transcriptomic analysis provides a broader view of how bioactive compounds affect bacterial physiology. Unlike RT-qPCR, which focuses on selected genes, transcriptomics evaluates global gene expression patterns and can reveal both expected and unexpected pathways affected by treatment (Lowe et al., 2017; Stark et al., 2019). This is especially important for anti-QS studies because compounds initially described as QS inhibitors may also modulate stress responses, oxidative metabolism, membrane transport, efflux pumps, secretion systems, iron uptake, amino acid metabolism, central carbon metabolism, or biofilm-associated pathways (Mukherjee & Bassler, 2019; Miranda et al., 2022; Kok et al., 2024).

Transcriptomic studies are useful for distinguishing narrow QS-targeted effects from broad physiological perturbations. A compound that specifically reduces expression of QS regulators and QS-controlled virulence genes while causing limited changes in housekeeping, ribosomal, and central metabolic genes may be interpreted differently from a compound that globally suppresses transcription across many essential pathways (Conesa et al., 2016; Love et al., 2014). The first profile is more compatible with targeted antivirulence activity, whereas the second may suggest general metabolic stress or toxicity. This distinction is important because the purpose of anti-QS strategies is usually to attenuate virulence without exerting strong selective pressure associated with bacterial killing (Mukherjee & Bassler, 2019; Shariati et al., 2024).

Transcriptomic approaches are also valuable for studying QS-related biofilm states. Biofilm formation is accompanied by large transcriptional reprogramming involving adhesion, matrix production, motility, transport, stress response, and metabolism (Wang et al., 2024; Abdelhamid & Yousef, 2024). A recent multi-omics study on *Streptococcus suis* compared planktonic and biofilm states and showed that combining transcriptomic and metabolomic analysis can identify genes and metabolites associated with biofilm formation and bacterial adaptation (Wang et al., 2024). Such designs are highly relevant for anti-QS research because they allow comparison of untreated planktonic cells, untreated biofilms, compound-treated planktonic cells, and compound-treated biofilms. This structure can reveal whether a compound prevents biofilm-specific transcriptional reprogramming or only reduces biomass through nonspecific effects (Wang et al., 2024; Zhao et al., 2025).

6.1.3 RNA sequencing

RNA sequencing is currently one of the most powerful approaches for investigating the global transcriptional response of pathogenic bacteria to bioactive compounds. It allows high-resolution detection of differentially expressed genes, operons, non-coding RNAs, regulatory networks, and pathway-level changes (Conesa et al., 2016; Stark et al., 2019). In QS-related studies, RNA-seq can identify whether a compound affects autoinducer synthesis, receptor expression, secretion systems, virulence genes, biofilm-associated genes, toxin genes, stress pathways, and metabolic adaptation (Lowe et al., 2017; Miranda et al., 2022).

RNA-seq is particularly useful when the mechanism of action of a compound is unknown. For example, a plant extract, nanoparticle, postbiotic fraction, fungal metabolite, or synthetic molecule may reduce several QS-related phenotypes, but the molecular target may not be clear (Liu et al., 2024; Shariati et al., 2024). RNA-seq can show whether the treatment primarily affects QS regulators, membrane stress, oxidative stress, iron metabolism, two-component systems, ribosomal activity, efflux pumps, or secondary metabolism (Conesa et al., 2016; Love et al., 2014; Kok et al., 2024). In LuxS/AI-2-related studies, transcriptomic profiling has been used to evaluate how *luxS* and AI-2-associated systems influence biofilm formation, pathogenicity, antibiotic resistance, and gene expression in pathogenic bacteria (Xie et al., 2024; Xu et al., 2025).

Nevertheless, RNA-seq requires rigorous experimental design. Biological replicates are essential, RNA integrity must be high, rRNA depletion or bacterial mRNA enrichment must be appropriate, sequencing depth must be sufficient, and bioinformatic analysis must be carefully controlled (Conesa et al., 2016; Stark et al., 2019). Differential expression analysis also requires robust statistical handling of dispersion, fold-change estimation, and multiple testing correction, for which tools such as DESeq2 are widely used (Love et al., 2014). The interpretation must also avoid overclaiming. Differential expression of QS-associated genes suggests transcriptional modulation, but it does not prove direct binding of the compound to QS receptors or direct inhibition of autoinducer synthesis. Therefore, RNA-seq should be followed by targeted validation using RT-qPCR and supported by phenotypic, proteomic, or metabolomic evidence (Bustin et al., 2009; Otto et al., 2014; Pellissier et al., 2021).

6.2 Target genes involved in quorum sensing

The selection of target genes is a decisive step in molecular anti-QS studies. Target genes must be chosen according to the bacterial species, the QS system involved, the virulence phenotype measured, and the expected mechanism of the tested compound (Papenfort & Bassler, 2016; Mukherjee & Bassler, 2019). It is methodologically weak to measure only one QS gene and draw broad conclusions about anti-QS activity, because QS systems are usually organized as networks involving signal synthases, receptors, response regulators, regulatory RNAs, downstream virulence genes, metabolic feedback loops, and environmental inputs (Papenfort & Bassler, 2016; Miranda et al., 2022).

In *P. aeruginosa*, the most frequently investigated QS genes include the Las, Rhl, and PQS systems, which regulate multiple virulence-related traits including elastase production, pyocyanin synthesis, rhamnolipid production, swarming, biofilm development, and secretion of extracellular factors (Miranda et al., 2022; Kristensen et al., 2024). In *S. aureus*, the agr system is central to toxin expression, dispersal-associated behavior, and secreted virulence factor production, although its relationship with biofilm formation is context-dependent (Lai et al., 2024; Yamazaki et al., 2024). In many Gram-negative and Gram-positive bacteria, *luxS* and AI-2-associated genes are investigated because AI-2 has been associated with interspecies communication, biofilm formation, motility, stress responses, antibiotic resistance, and pathogenicity (Pereira et al., 2013; Juszczuk-Kubiak, 2024).

6.2.1 *lasI/lasR*

The *lasI/lasR* system is one of the best-characterized QS circuits in *P. aeruginosa*. *lasI* encodes the synthase responsible for producing the autoinducer N-3-oxo-dodecanoyl homoserine lactone, while *lasR* encodes the transcriptional regulator that responds to this signal and activates downstream QS-controlled genes (Papenfort & Bassler, 2016; Miranda et al., 2022). The Las system has traditionally been viewed as a high-level regulator in the QS hierarchy of *P. aeruginosa*, influencing elastase production, protease activity, biofilm formation, secondary metabolism, and other virulence-associated traits (Miranda et al., 2022; Ren et al., 2024).

In anti-QS studies, reduced expression of *lasI* may suggest impaired autoinducer synthesis, whereas reduced expression of *lasR* may indicate that the regulatory receptor itself is affected. However, interpretation is not always straightforward, because clinical isolates and stress-adapted populations may show regulatory rewiring, altered QS hierarchy, or compensatory activation of alternative pathways (Mukherjee & Bassler, 2019; Miranda et al., 2022). Some compounds may also reduce Las-

regulated phenotypes without directly targeting LasR, for example by interfering with downstream virulence protein activity, secretion, metabolism, or efflux-associated regulation (Ren et al., 2024; Kristensen et al., 2024).

A strong experimental design may compare the effect of a compound on wild-type strains, QS mutants, and clinical isolates with different QS backgrounds. If a compound reduces virulence in a wild-type strain but has little effect in a *lasR* mutant, this may suggest Las-dependent activity. If the compound remains active in Las-defective isolates, other systems may be involved (Mukherjee & Bassler, 2019; Miranda et al., 2022). This approach is particularly important because clinical *P. aeruginosa* isolates may not always follow the classical QS hierarchy observed in laboratory strains (Miranda et al., 2022).

6.2.2 *rhlI/rhlR*

The *rhlI/rhlR* system is another major QS circuit in *P. aeruginosa*. *rhlI* encodes the synthase responsible for producing N-butyryl homoserine lactone, while *rhlR* encodes the corresponding transcriptional regulator (Papenfort & Bassler, 2016; Miranda et al., 2022). The Rhl system controls several virulence-related traits, including rhamnolipid production, swarming motility, pyocyanin production, biofilm development, and secreted factors (Miranda et al., 2022; Kristensen et al., 2024).

For anti-QS studies, *rhlI* and *rhlR* expression should be measured when phenotypic assays show changes in swarming, rhamnolipid-associated behavior, pyocyanin production, or biofilm architecture (Miranda et al., 2022; Liu et al., 2024). A compound that reduces swarming motility and pyocyanin while downregulating *rhlI/rhlR* provides stronger evidence of Rhl-associated QS modulation than phenotype alone (Liu et al., 2024; Shariati et al., 2024). However, because QS circuits operate within wider regulatory networks, measuring both *rhlI* and *rhlR*, together with downstream genes such as *rhlA*, *phz* genes, or *lasB*, is preferable to measuring only one regulator (Papenfort & Bassler, 2016; Miranda et al., 2022).

This is especially important when testing chemically complex compounds such as plant extracts, fungal metabolites, nanoparticles, postbiotic preparations, or natural compounds that may affect multiple regulatory layers at once (Liu et al., 2024; Shariati et al., 2024). In such cases, downregulation of *rhlI/rhlR* must be interpreted alongside growth data, phenotypic assays, and ideally signal molecule quantification.

6.2.3 *agr* systems

The accessory gene regulator system is the central QS system controlling several virulence traits in *S. aureus*. It is composed of the autoinducing peptide signaling machinery and regulatory components that influence the expression of toxins, exoenzymes, immune evasion factors, and biofilm-associated traits (Lai et al., 2024; Yamazaki et al., 2024). In many experimental contexts, agr activation promotes secreted virulence factor production and dispersal-like behavior, while reduced agr activity may be associated with stronger surface attachment and biofilm persistence (Yamazaki et al., 2024).

In anti-QS studies involving *S. aureus*, the most relevant molecular targets may include *agrA*, *agrB*, *agrC*, *agrD*, *RNAIII*, and downstream toxin genes such as *hla* or genes encoding phenol-soluble modulins (Lai et al., 2024; Yamazaki et al., 2024). If a compound reduces hemolysis, toxin production, or secreted protease activity, agr gene expression can help determine whether this effect is linked to QS modulation. Recent studies on *S. aureus* isolates have shown the relevance of agr typing, virulence-associated gene profiling, and biofilm evaluation when analyzing the relationship between QS, virulence, and clinical behavior (Lai et al., 2024).

At the same time, agr inhibition is not always universally beneficial. In some infection contexts, agr-defective or agr-repressed phenotypes may favor persistent biofilm-associated infection, whereas agr activation may promote toxin production and tissue damage (Yamazaki et al., 2024). Therefore, an anti-agr effect should not automatically be presented as therapeutically favorable without considering whether the compound reduces damaging toxin production, enhances biofilm persistence, or alters bacterial clearance. The best studies evaluate agr-associated gene expression together with hemolysis, cytotoxicity, biofilm formation, adhesion, and, when possible, host-cell responses (Lai et al., 2024; Yamazaki et al., 2024).

6.2.4 *luxS* and AI-2 associated genes

The *luxS*/AI-2 system is widely studied because AI-2 has been associated with interspecies communication and regulation of diverse bacterial behaviors, including biofilm formation, motility, metabolism, stress tolerance, antibiotic resistance, and virulence (Pereira et al., 2013; Juszczuk-Kubiak, 2024). *luxS* encodes an enzyme involved in the activated methyl cycle and AI-2 production, which creates an important interpretive challenge: changes in *luxS* expression or *luxS* deletion may affect both signaling and central metabolism (Pereira et al., 2013; Juszczuk-Kubiak, 2024).

In pathogenic bacteria, studies have used *luxS* mutants, AI-2 activity assays, gene expression analysis, and transcriptomic profiling to investigate the role of AI-2/LuxS in biofilm formation, pathogenicity, stress adaptation, and antibiotic resistance (Li et al., 2007; Xie et al., 2024; Xu et al., 2025). In *Escherichia coli*, AI-2/LsrR signaling has been connected to small RNA regulation and biofilm architecture, showing that AI-2-associated pathways may influence complex bacterial behaviors beyond simple signal accumulation (Li et al., 2007). More recent studies have evaluated the contribution of LuxS/AI-2 systems to virulence-associated traits and transcriptomic changes in pathogenic bacteria, reinforcing their methodological relevance in anti-QS investigations (Xie et al., 2024; Xu et al., 2025).

For anti-QS research, *luxS* expression is especially relevant when the studied pathogen is known to use AI-2-associated regulation or when phenotypes such as biofilm formation, motility, adhesion, or stress tolerance are affected by the tested compound (Pereira et al., 2013; Juszczuk-Kubiak, 2024). However, because LuxS is metabolically connected to the activated methyl cycle, it is methodologically safer to combine *luxS* expression with AI-2 quantification, transcriptomic analysis, and phenotypic validation rather than concluding that a compound is an AI-2 inhibitor based on *luxS* expression alone (Pereira et al., 2013; Xu et al., 2025).

6.3 Proteomic approaches

Proteomic approaches provide information that cannot be obtained from transcriptomics alone. While gene expression analysis measures mRNA levels, proteomics evaluates the abundance, modification, localization, secretion, and sometimes functional state of proteins (Otto et al., 2014). This is particularly important in QS-related virulence because many key factors are secreted proteins, extracellular enzymes, toxins, adhesins, transporters, and stress-response proteins (Solis et al., 2014; Wu et al., 2019).

Proteomics is especially useful when studying extracellular virulence factors. In pathogens such as *P. aeruginosa* and *S. aureus*, QS influences secreted enzymes, toxins, proteases, immune evasion proteins, and biofilm-associated proteins (Miranda et al., 2022; Yamazaki et al., 2024). Therefore, proteomic analysis can determine whether phenotypic reductions in protease activity, hemolysis, elastase activity, or biofilm formation correspond to changes in protein abundance (Otto et al., 2014; Solis et al., 2014). Proteomic studies have also shown that bacterial surface and secreted protein profiles vary according to growth conditions, which is directly relevant for interpreting virulence expression and experimental reproducibility (Solis et al., 2014).

6.3.1 LC-MS/MS

Liquid chromatography–tandem mass spectrometry is one of the most powerful methods for bacterial proteomic analysis. It allows identification and relative or absolute quantification of large numbers of proteins from whole-cell lysates, membrane fractions, biofilm extracts, or culture supernatants (Otto et al., 2014; Wu et al., 2019). In anti-QS studies, LC-MS/MS can be used to determine whether a bioactive compound reduces the abundance of QS-regulated proteins such as elastases, proteases, phenazine biosynthesis enzymes, rhamnolipid-associated enzymes, toxins, adhesins, secretion system components, or stress-response proteins (Solis et al., 2014; Wu et al., 2019).

The main advantage of LC-MS/MS is its depth and specificity. It can reveal whether a compound affects only a small group of virulence-associated proteins or causes broad proteomic disruption (Otto et al., 2014). This distinction is critical. A selective decrease in secreted virulence factors without major reduction in core metabolic proteins may support an antivirulence interpretation. In contrast, broad suppression of ribosomal proteins, central metabolic enzymes, and essential cellular machinery may indicate general toxicity or growth inhibition (Otto et al., 2014; Amado et al., 2026).

LC-MS/MS also helps resolve discrepancies between gene expression and phenotype. For example, a compound may not strongly reduce *lasB* transcription but may reduce extracellular LasB protein abundance through altered secretion or protein stability; conversely, mRNA levels may decrease without measurable reduction in extracellular enzyme activity if the protein is stable in the medium (Otto et al., 2014; Solis et al., 2014). Therefore, LC-MS/MS provides a bridge between transcriptional regulation and functional phenotypic outcomes.

6.3.2 MALDI-TOF analysis

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry is widely known in clinical microbiology for bacterial identification, but it can also provide useful protein fingerprinting information. In the context of anti-QS or antivirulence research, MALDI-TOF may be used to detect global changes in bacterial protein profiles, compare treated and untreated cells, or identify shifts in abundant proteins under defined conditions (Flores-Flores et al., 2025). Its major strengths are speed, relatively low sample requirement, and suitability for comparative profiling.

However, MALDI-TOF is generally less comprehensive than LC-MS/MS for deep proteomic discovery. It is more appropriate as a rapid fingerprinting or classification tool than as a complete method for identifying QS-regulated proteomic changes (Otto et al., 2014; Flores-Flores et al., 2025). It may help detect whether a compound causes major alterations in bacterial protein profiles, but it usually cannot replace LC-MS/MS when the objective is to identify specific virulence proteins or secreted factors. Therefore, MALDI-TOF should be positioned as a complementary method rather than the central proteomic approach for mechanistic anti-QS studies (Flores-Flores et al., 2025).

6.3.3 Secretome profiling

Secretome profiling is particularly relevant for QS research because many QS-regulated virulence factors are secreted into the extracellular environment. These include proteases, elastases, lipases, toxins, hemolysins, immune-modulating proteins, siderophore-associated proteins, and enzymes involved in tissue damage or nutrient acquisition (Solis et al., 2014; Miranda et al., 2022; Yamazaki et al., 2024). A bioactive compound that reduces secreted virulence factors without strongly affecting bacterial growth may have strong antivirulence potential.

Secretome analysis generally involves collecting culture supernatants under standardized conditions, removing bacterial cells, concentrating extracellular proteins, and analyzing them by SDS-PAGE, LC-MS/MS, or targeted immunological methods (Solis et al., 2014; Otto et al., 2014). The design must carefully control for bacterial density because reduced secreted protein abundance may simply reflect lower cell numbers. Protein degradation, medium composition, precipitation, and compound interference must also be considered (Solis et al., 2014; Wu et al., 2019).

Secretome profiling can be highly informative when linked to phenotypic enzyme assays. If a compound reduces elastase activity, secretome LC-MS/MS can determine whether LasB abundance is actually reduced. If hemolysis decreases, secretome analysis can evaluate whether alpha-hemolysin or other toxins are reduced. If protease activity is lower, extracellular protease abundance can be measured (Solis et al., 2014; Otto et al., 2014). This makes secretome profiling one of the most biologically relevant omics approaches for evaluating QS-related virulence attenuation.

6.4 Metabolomic approaches

Metabolomics provides a functional view of bacterial physiology by measuring small molecules involved in signaling, metabolism, stress adaptation, virulence, and intercellular communication (Depke et al., 2020; Pellissier et al., 2021). In anti-QS studies, metabolomics is particularly important because QS depends on chemical signaling molecules, and many QS-regulated virulence factors are themselves metabolites or metabolite-derived products, including AHLs, quinolones, phenazines, rhamnolipids, siderophores, indole-related molecules, AI-2-associated compounds, organic acids, amino acid derivatives, and secondary metabolites (Pellissier et al., 2021; Abdelhamid & Yousef, 2024).

The main advantage of metabolomics is that it can reveal how a compound changes bacterial chemical behavior. A treatment may reduce transcription of QS genes, but metabolomics can show whether this actually leads to reduced autoinducer accumulation or altered production of virulence-associated metabolites (Depke et al., 2020; Pellissier et al., 2021). It can also reveal unexpected metabolic shifts, such as changes in amino acid metabolism, carbon utilization, oxidative stress metabolites, membrane lipids, or iron-scavenging molecules (Abdelhamid & Yousef, 2024; Vinayavekhin et al., 2024; Zhao et al., 2025). Multi-omics studies increasingly show that bacterial biofilm formation and virulence adaptation involve coordinated changes in genes and metabolites rather than isolated regulatory events (Wang et al., 2024; Zhao et al., 2025).

6.4.1 LC-MS-based metabolomics

LC-MS-based metabolomics is one of the most suitable approaches for detecting QS signaling molecules and virulence-associated metabolites because it can analyze compounds with diverse polarity, molecular weight, and chemical structure (Depke et al., 2020; Pellissier et al., 2021). In *P. aeruginosa*, LC-MS can be used to detect and quantify AHLs, quinolone signals such as PQS and HHQ, phenazines, rhamnolipids, and other secondary metabolites involved in virulence and community behavior (Pellissier et al., 2021; Depke et al., 2020).

In anti-QS studies, this approach can determine whether a compound reduces signal molecule accumulation or downstream metabolite production. The value of LC-MS metabolomics is particularly high when the compound being tested is chemically complex, as in the case of plant extracts, postbiotic preparations, fermented products, fungal metabolites, or nanoparticle-associated metabolites (Liu et al., 2024; Shariati et al., 2024; Zhao et al., 2025). Untargeted LC-MS can reveal broad metabolic

changes, while targeted LC-MS/MS can quantify specific autoinducers or virulence metabolites (Depke et al., 2020; Pellissier et al., 2021).

However, LC-MS metabolomics requires careful sample preparation. QS signals may be unstable, hydrophobic, low-abundance, or sensitive to extraction conditions (Pellissier et al., 2021). Culture medium can introduce background signals, plant extracts can contain overlapping metabolites, and nanoparticles may adsorb signaling molecules or interfere with extraction. Therefore, medium-only controls, compound-only controls, extraction blanks, internal standards, and normalization to bacterial density are essential (Pellissier et al., 2021; Zhao et al., 2025). Without these controls, reduced signal detection may be caused by chemical adsorption, degradation, or matrix effects rather than true inhibition of QS production.

6.4.2 GC-MS analysis

Gas chromatography–mass spectrometry is useful for detecting volatile, semi-volatile, and derivatized metabolites. In bacterial virulence research, GC-MS can be used to investigate metabolic changes related to organic acids, fatty acids, amino acid derivatives, volatile compounds, and other small molecules (Vinayavekhin et al., 2024; Zhao et al., 2025). Although GC-MS is less commonly used than LC-MS for direct detection of many QS autoinducers, it can provide valuable information about metabolic reprogramming induced by bioactive compounds (Vinayavekhin et al., 2024).

GC-MS may be particularly useful when studying plant-derived compounds, essential oils, volatile antimicrobials, and bacterial volatile organic compounds. If an essential oil or volatile-rich extract reduces biofilm formation, pigment production, or motility, GC-MS can help characterize the active chemical constituents and evaluate whether bacterial volatile or central metabolic profiles are altered after treatment (Vinayavekhin et al., 2024; Zhao et al., 2025). In this way, GC-MS can contribute both to compound characterization and to understanding bacterial metabolic response.

The limitation of GC-MS is that many QS signals are not directly compatible with gas chromatography unless derivatized, and thermolabile molecules may degrade during analysis. Therefore, GC-MS should be selected according to the chemical nature of the molecules of interest. For AHLs, quinolones, phenazines, and many siderophore-related molecules, LC-MS/MS is generally more appropriate; for volatile compounds and derivatized metabolites, GC-MS remains highly useful (Pellissier et al., 2021; Vinayavekhin et al., 2024).

6.4.3 Detection and quantification of signaling molecules

Direct detection and quantification of signaling molecules represent one of the strongest forms of evidence in anti-QS studies. If a compound reduces QS-regulated phenotypes and also decreases the concentration of autoinducers, the argument for QS interference becomes stronger (Pellissier et al., 2021; Liu et al., 2024). In Gram-negative bacteria, the most frequently investigated signals include AHLs, quinolones, and AI-2-associated molecules, whereas in Gram-positive bacteria, autoinducing peptides are central to systems such as agr in *S. aureus* (Papenfort & Bassler, 2016; Pereira et al., 2013; Yamazaki et al., 2024).

Several approaches can be used for signal detection. Biosensor strains provide functional detection of signaling activity, such as AHL-mediated induction of reporter phenotypes. LC-MS/MS provides chemical detection and quantification of defined signals. Reporter assays can show whether extracellular extracts from treated cultures retain QS-inducing activity (Pellissier et al., 2021). Together, these methods can distinguish between reduced signal production, signal degradation, receptor antagonism, and downstream transcriptional interference (Papenfort & Bassler, 2016; Mukherjee & Bassler, 2019).

This distinction is crucial. A compound may reduce virulence without reducing autoinducer concentration if it blocks receptor activation or downstream signaling. Another compound may reduce extracellular signal concentration by inhibiting autoinducer synthesis, chemically degrading the signal, adsorbing it, or altering membrane transport (Papenfort & Bassler, 2016; Pellissier et al., 2021). Nanoparticles, for example, may reduce detectable signaling molecules through adsorption or catalytic degradation rather than transcriptional inhibition, while plant extracts may contain molecules that interfere with biosensor responses or LC-MS ionization (Shariati et al., 2024; Zhao et al., 2025). Therefore, direct signal quantification must include compound-only controls, spiked recovery experiments, internal standards, and, when possible, comparison with purified signals (Pellissier et al., 2021).

The strongest methodological interpretation is obtained when signaling molecule quantification is integrated with gene expression and phenotypic data. In *P. aeruginosa*, a compound that reduces *lasI/rhlI* expression, lowers AHL and quinolone signal levels, decreases pyocyanin and elastase production, and does not substantially inhibit growth provides a coherent anti-

QS profile (Miranda et al., 2022; Pellissier et al., 2021; Liu et al., 2024). In *S. aureus*, a compound that reduces agr activity, lowers toxin expression, decreases hemolysis, and does not merely suppress growth can be interpreted as a stronger antivirulence candidate (Lai et al., 2024; Yamazaki et al., 2024). In LuxS/AI-2 systems, reduced AI-2 activity should be interpreted alongside *luxS* expression, growth controls, metabolic data, and relevant phenotypes because LuxS has both signaling and metabolic roles (Pereira et al., 2013; Juszczuk-Kubiak, 2024; Xu et al., 2025).

Integrated interpretation of molecular and omics data

Molecular and omics approaches should not be treated as isolated layers but as complementary levels of evidence. RT-qPCR provides targeted confirmation of selected genes, RNA-seq reveals global transcriptional reprogramming, proteomics determines whether transcriptional changes are reflected at the protein level, secretome profiling focuses on extracellular virulence factors, and metabolomics shows whether signaling molecules, virulence metabolites, and metabolic pathways are altered (Bustin et al., 2009; Conesa et al., 2016; Otto et al., 2014; Pellissier et al., 2021). Together, these approaches allow the investigator to distinguish between true QS interference, general antibacterial stress, metabolic toxicity, secretion impairment, and assay-level artifacts (Mukherjee & Bassler, 2019; Depke et al., 2020; Zhao et al., 2025).

A robust anti-QS study should ideally follow a progressive logic. First, sub-MIC concentrations are established and phenotypic assays identify reduced virulence traits. Second, RT-qPCR evaluates key QS and virulence genes linked to those phenotypes. Third, RNA-seq may be used when the mechanism is unclear or when broad regulatory effects are suspected. Fourth, proteomics and secretome profiling verify whether reduced gene expression translates into reduced virulence protein abundance. Fifth, metabolomics and signal quantification determine whether autoinducers and QS-regulated metabolites are directly affected (Taylor et al., 2019; Stark et al., 2019; Otto et al., 2014; Pellissier et al., 2021).

This layered approach is particularly important for complex bioactive compounds. Plant extracts, medicinal compounds, postbiotics, nanoparticles, antimicrobial peptides, fungal metabolites, and synthetic small molecules may act through multiple mechanisms simultaneously (Liu et al., 2024; Shariati et al., 2024; Zhao et al., 2025). A plant extract may contain QS receptor antagonists, membrane-active compounds, antioxidants, and iron-chelating molecules. A nanoparticle may inhibit growth at higher concentrations, adsorb signaling molecules, generate oxidative stress, and reduce biofilm formation. A postbiotic preparation may contain organic acids, peptides, biosurfactants, enzymes, and metabolites that act on several virulence pathways. Without molecular and omics validation, it is difficult to determine whether the observed phenotype is truly QS-related (Pellissier et al., 2021; Mbarga et al., 2021 ; Arsene et al., 2023 ; Mouafo et al., 2023 ; Abdelhamid & Yousef, 2024; Zhao et al., 2025).

The most convincing conclusion is therefore not based on a single molecular marker but on convergence. When a bioactive compound reduces QS-related phenotypes at non-growth-inhibitory concentrations, downregulates relevant QS and virulence genes, decreases virulence-associated proteins in the secretome, and lowers the abundance or activity of signaling molecules, the evidence for anti-QS or antivirulence activity becomes substantially stronger (Bustin et al., 2009; Otto et al., 2014; Pellissier et al., 2021; Liu et al., 2024). Conversely, if phenotypic inhibition occurs without corresponding molecular changes, the investigator should consider alternative mechanisms such as direct enzyme inhibition, matrix disruption, iron chelation, membrane effects, metabolic stress, or assay interference (Mukherjee & Bassler, 2019; Zhao et al., 2025).

Thus, molecular and omics approaches transform anti-QS research from a screening-based field into a mechanistically grounded discipline. They allow researchers to move beyond the statement that a compound “reduces biofilm” or “inhibits pigment production” toward a more precise explanation of how bacterial communication, virulence regulation, secretion, and metabolism are altered (Mukherjee & Bassler, 2019; Otto et al., 2014; Pellissier et al., 2021). This level of methodological rigor is essential for identifying compounds with genuine antivirulence potential and for distinguishing promising QS-targeting candidates from nonspecific antimicrobial or assay-interfering agents.

Although phenotypic observations provide valuable preliminary information, molecular validation is required to confirm that a compound genuinely affects quorum sensing pathways. Modern anti-QS research increasingly relies on molecular and omics technologies to investigate signaling mechanisms at multiple biological levels. The principal molecular and omics approaches used in anti-QS investigations are presented in Table 4.

Table 4. Molecular and omics techniques used in anti-QS studies

Molecular / omics technique	Main objective in anti-QS studies	Typical targets or outputs	Main contribution to anti-QS interpretation	Key methodological precautions	References
RT-qPCR analysis of QS-related genes	Quantify transcriptional modulation of QS regulators, autoinducer synthases, and QS-controlled virulence genes	lasI, lasR, rhlI, rhlR, pqsA, pqsR, luxS, sdiA, abaI, abaR, agrA, agrC, RNAIII, gelE, virulence and biofilm-associated genes	Demonstrates whether a bioactive compound downregulates QS-associated transcription at sub-inhibitory concentrations	Requires validated reference genes, growth-phase matching, RNA integrity control, no-RT controls, primer efficiency testing, and MIQE-compliant reporting	Bustin et al., 2009; Taylor et al., 2019; Papenfort & Bassler, 2016; Mukherjee & Bassler, 2019; Miranda et al., 2022
RT-qPCR analysis of downstream virulence genes	Verify whether phenotypic attenuation is associated with transcriptional repression of virulence determinants	Genes involved in toxins, proteases, elastase, hemolysins, siderophores, motility, EPS synthesis, secretion systems, and adhesion	Connects reduced virulence phenotypes to molecular regulation rather than simple assay interference	Should be interpreted together with growth curves, phenotypic assays, and protein or metabolite-level validation	Bustin et al., 2009; Taylor et al., 2019; Mukherjee & Bassler, 2019; Shariati et al., 2024
RNA sequencing / transcriptomics	Provide global transcriptional profiling of treated versus untreated bacteria	QS regulons, virulence pathways, stress-response genes, metabolism, secretion systems, biofilm genes, transporters, two-component systems	Identifies whether the compound selectively affects QS-related networks or broadly disrupts bacterial physiology	Requires biological replicates, RNA quality control, growth-phase standardization, appropriate normalization, pathway enrichment, and validation by RT-qPCR	Stark et al., 2019; Pellissier et al., 2021; Mukherjee & Bassler, 2019; Wang et al., 2024; Zhao et al., 2025
Comparative transcriptomic pathway analysis	Determine whether anti-QS effects are pathway-specific or part of a broader stress response	QS pathways, biofilm pathways, oxidative stress, iron acquisition, efflux systems, secretion pathways, metabolic networks	Helps distinguish targeted QS inhibition from nonspecific metabolic suppression or antimicrobial stress	Differentially expressed genes should not be overinterpreted without phenotypic and biochemical confirmation	Pellissier et al., 2021; Abdelhamid & Yousef, 2024; Zhao et al., 2025; Fernandes et al., 2024
Reporter gene assays	Monitor QS promoter activity or QS pathway activation in real time or endpoint formats	Fluorescent, luminescent, or colorimetric reporters under QS-regulated promoters	Provides functional evidence that a compound interferes with QS-dependent transcription or signal perception	Reporter inhibition must be separated from growth inhibition, fluorescence quenching, compound color, cytotoxicity, and metabolic interference	Papenfort & Bassler, 2016; Mukherjee & Bassler, 2019; Naga et al., 2024; Hetta et al., 2024
QS biosensor assays	Detect inhibition, degradation, or neutralization of QS signals	AHLs, AI-2-like signals, quinolone signals, peptide-mediated QS outputs depending on biosensor system	Useful for preliminary screening and functional confirmation of signal interference	Biosensor results require growth controls, signal rescue experiments, solvent controls, and confirmation in pathogenic models	Warrier et al., 2021; Naga et al., 2024; Hetta et al., 2024; D'Aquila et al., 2024
Signal rescue / complementation assays	Test whether addition of exogenous autoinducers restores QS-regulated phenotypes	AHLs, AI-2-related signals, AIPs, PQS/HHQ or other relevant signals	Strengthens evidence that the compound acts on QS signaling rather than on downstream phenotypes only	Signal concentration, timing of addition, compound–signal interaction, and strain-specific responsiveness must be controlled	Papenfort & Bassler, 2016; Mukherjee & Bassler, 2019; Miranda et al., 2022; Hetta et al., 2024
Targeted autoinducer quantification	Directly measure whether treatment reduces QS signal accumulation	AHLs, PQS, HHQ, AI-2-associated metabolites, AIPs, indole-related signals	Provides direct chemical evidence of signal inhibition, degradation, adsorption, or altered production	Requires analytical standards, calibration curves, extraction validation, matrix controls, and normalization to biomass or cell density	Depke et al., 2020; Pellissier et al., 2021; Stark et al., 2019; Otto et al., 2014
LC-MS-based signalomics	Identify and quantify QS signaling molecules and	AHLs, quinolones, phenazines, rhamnolipids, siderophores, organic	Links anti-QS activity to changes in the chemical communication landscape of bacteria	Requires careful sample preparation, internal standards, compound annotation, ion suppression controls,	Depke et al., 2020; Pellissier et al., 2021; Abdelhamid & Yousef, 2024; Liu et al., 2024

	signal-related metabolites	acids, secondary metabolites		and distinction between signal reduction and growth reduction	
HPLC or UHPLC profiling	Quantify selected QS signals, pigments, metabolites, or compound-derived products	Pyocyanin, phenazines, quinolones, plant-derived metabolites, postbiotic metabolites, QS-associated small molecules	Provides targeted chemical confirmation of reduced signal or virulence-associated metabolite production	Peak identity should be confirmed by standards or MS when possible; compound interference and extraction recovery must be evaluated	Pellissier et al., 2021; Depke et al., 2020; Shariati et al., 2024; Liu et al., 2024
LC-MS/MS proteomics	Quantify global or targeted protein-level changes induced by anti-QS treatment	QS-regulated proteins, enzymes, toxins, adhesins, secretion proteins, stress-response proteins, biofilm-associated proteins	Determines whether transcriptional or phenotypic changes translate into altered protein abundance	Requires sufficient biological replicates, standardized protein extraction, digestion quality control, normalization, and database reliability	Otto et al., 2014; Solis et al., 2014; Wu et al., 2019; Pellissier et al., 2021
Secretome profiling	Analyze extracellular proteins released or secreted by bacteria under treatment	Elastase, proteases, hemolysins, toxins, secreted enzymes, adhesins, extracellular matrix-associated proteins	Particularly relevant because many QS-regulated virulence factors are secreted proteins	Must control protein degradation, medium composition, precipitation bias, compound interference, and differences in bacterial growth	Otto et al., 2014; Solis et al., 2014; Wu et al., 2019
Western blotting	Validate changes in selected QS-regulated proteins or toxins	Specific toxins, enzymes, regulatory proteins, surface proteins, secreted virulence factors	Provides targeted protein-level confirmation of transcriptomic or proteomic results	Requires validated antibodies, loading controls, normalization, and distinction between reduced expression and reduced secretion	Otto et al., 2014; Solis et al., 2014
ELISA-based toxin or protein quantification	Quantify selected secreted toxins, enzymes, or virulence-associated proteins	Hemolysins, enterotoxins, exotoxins, proteases, inflammatory or host-response markers in infection models	Supports the antivirulence interpretation when toxin reduction occurs without growth inhibition	Compound interference, antigen masking, sample dilution, matrix effects, and viability controls must be considered	Otto et al., 2014; Podkowik et al., 2024; Beenken et al., 2025
Metabolomics	Characterize global metabolic changes associated with QS inhibition or antivirulence activity	Autoinducers, phenazines, rhamnolipids, siderophores, amino acids, lipids, organic acids, secondary metabolites	Reveals whether treatment reshapes bacterial metabolism toward a less virulent state	Requires standardized extraction, normalization, annotation confidence, pathway analysis, and separation of QS-specific effects from general metabolic stress	Depke et al., 2020; Pellissier et al., 2021; Abdelhamid & Yousef, 2024; Vinayavekhin et al., 2024; Zhao et al., 2025
GC-MS-based metabolite profiling	Analyze volatile, derivatized, or small metabolic compounds affected by treatment	Organic acids, fatty acid derivatives, volatile metabolites, central metabolism intermediates	Complements LC-MS by detecting metabolite classes not optimally covered by LC-MS	Derivatization, volatility, extraction efficiency, and compound stability must be controlled	Depke et al., 2020; Pellissier et al., 2021; Abdelhamid & Yousef, 2024
NMR-based metabolomics	Provide reproducible, quantitative metabolic fingerprints with minimal sample bias	High-abundance metabolites, organic acids, amino acids, fermentation products, metabolic pathway markers	Useful for confirming broad metabolic shifts and comparing treated and untreated conditions	Less sensitive than MS-based methods for low-abundance signals; requires adequate concentration and careful spectral interpretation	Depke et al., 2020; Pellissier et al., 2021
Multi-omics integration	Combine transcriptomics, proteomics, metabolomics, phenotypic data, and signal quantification	QS gene expression, protein abundance, metabolite production, biofilm outputs, toxin production, motility, signaling molecules	Provides the strongest mechanistic evidence by showing convergence across biological layers	Requires harmonized sampling time points, compatible experimental design, robust bioinformatics, and cautious causal interpretation	Pellissier et al., 2021; Wang et al., 2024; Abdelhamid & Yousef, 2024; Zhao et al., 2025
Network pharmacology	Map compound–target–pathway	QS regulators, virulence pathways, host-pathogen interaction nodes,	Useful for complex mixtures such as plant extracts, essential oils,	Predicted networks must be experimentally validated; network	Wu et al., 2022; Hetta et al., 2024; Fernandes et al., 2024

	relationships in anti-QS and antivirulence research	biofilm networks, inflammatory pathways	marine extracts, nanoparticles, and postbiotics	centrality does not prove biological causality	
Systems biology modeling	Interpret QS inhibition as part of integrated bacterial regulatory networks	QS circuits, metabolism, stress responses, secretion, biofilm development, cyclic-di-GMP, iron acquisition	Helps identify whether a compound targets QS directly or affects upstream/downstream regulatory modules	Model outputs depend on data quality, pathway annotation, and experimental validation	Wu et al., 2022; Hetta et al., 2024; Pellissier et al., 2021; Zhao et al., 2025
Molecular docking	Predict binding of bioactive compounds to QS receptors or enzymes	LasR, RhlR, PqsR/MvfR, CviR, LuxR-type regulators, AgrC/AgrA-associated targets, signal synthases	Helps prioritize candidate compounds and propose receptor-antagonism mechanisms	Docking alone is insufficient; results require ADMET evaluation, molecular dynamics when possible, and experimental validation	D'Aquila et al., 2024; Hetta et al., 2024; Ramasamy et al., 2023
Molecular dynamics simulation	Evaluate stability of predicted ligand–QS target interactions over time	QS receptor–ligand complexes, binding-pocket stability, interaction energies, conformational changes	Strengthens computational support for receptor-targeted anti-QS hypotheses	Simulation parameters, force fields, time scale, solvent model, and biological relevance must be clearly reported	Ramasamy et al., 2023; D'Aquila et al., 2024
AI-assisted molecular screening	Predict anti-QS candidates using chemical descriptors, molecular fingerprints, QS assay data, toxicity profiles, and target information	Natural compounds, synthetic analogues, marine metabolites, phytochemicals, repurposed molecules	Accelerates discovery and prioritization of candidate QS inhibitors	Models require curated datasets, clear definitions of QS inhibition, interpretability, and laboratory validation	Ramasamy et al., 2023; Wu et al., 2022; Fernandes et al., 2024
Mutant and complementation analysis	Verify whether observed anti-QS effects depend on a specific QS pathway	QS-deficient mutants, complemented strains, overexpression strains, signal synthase or receptor mutants	Provides causal evidence that a compound acts through a QS-linked pathway	Mutant phenotypes may be pleiotropic; complementation and growth-matched controls are essential	Papenfort & Bassler, 2016; Mukherjee & Bassler, 2019; Miranda et al., 2022
Genomic or strain-level characterization	Confirm the presence, integrity, or variation of QS-related genes in tested strains	QS loci, virulence genes, biofilm genes, resistance genes, strain-specific regulatory variants	Avoids misinterpretation when strains lack functional QS systems or carry mutations affecting QS outputs	Particularly important for clinical isolates, which may differ from reference strains in QS gene functionality	Miranda et al., 2022; Zhang et al., 2025; Sun et al., 2021; Pumirat et al., 2024
Bioinformatic pathway enrichment	Interpret omics datasets in relation to QS, virulence, metabolism, and biofilm regulation	KEGG-like pathways, virulence networks, regulatory modules, metabolic pathways, stress responses	Helps identify biological processes altered by anti-QS treatment	Enrichment results should be interpreted as hypothesis-generating unless confirmed experimentally	Stark et al., 2019; Pellissier et al., 2021; Wang et al., 2024; Zhao et al., 2025
Integrated phenotypic–molecular validation	Combine phenotypic assays with gene expression, protein analysis, metabolomics, and signal quantification	Biofilm, motility, pigment, enzyme activity, toxins, QS genes, proteins, autoinducers	Represents the most rigorous approach for distinguishing true QS inhibition from nonspecific antimicrobial or antibiofilm effects	Requires coherent experimental design, matched time points, sub-MIC conditions, and convergence across independent endpoints	Bustin et al., 2009; Otto et al., 2014; Pellissier et al., 2021; Liu et al., 2024; Hetta et al., 2024

7. Biosensor-Based and Advanced Analytical Approaches

The investigation of bioactive compounds targeting quorum sensing requires methodological approaches capable of detecting subtle modifications in bacterial communication without confusing genuine quorum-quenching effects with simple growth inhibition or nonspecific toxicity. Although classical phenotypic assays remain indispensable for evaluating biofilm formation, motility, pigment production, enzyme secretion, toxin release, and other virulence-associated traits, these approaches usually measure downstream consequences of quorum sensing rather than the signaling process itself. Biosensor-based and advanced analytical methods occupy a strategic position in this context because they allow researchers to detect, quantify, localize, or functionally interpret quorum-sensing signals and quorum-sensing inhibition at earlier and more mechanistic levels. Whole-cell biosensors, cell-free systems, reporter strains, high-throughput screening platforms, and microfluidic devices therefore provide complementary layers of evidence when assessing whether antibacterial agents, plant-derived compounds, essential oils, nanoparticles, postbiotics, or synthetic molecules interfere with quorum sensing-related virulence regulation (Miller & Gilmore, 2020; Chen et al., 2023; Green et al., 2025).

Unlike conventional antibacterial assays, quorum sensing biosensors are not primarily designed to determine whether a compound kills or inhibits bacterial growth. Their value lies in their ability to translate the presence or inhibition of signaling molecules into measurable readouts such as pigmentation, fluorescence, luminescence, absorbance changes, or reporter gene expression. This distinction is methodologically important because many anti-quorum sensing compounds are expected to act at sub-inhibitory concentrations, attenuating virulence without necessarily exerting bactericidal or bacteriostatic pressure. In this sense, biosensor assays can help distinguish between compounds that suppress quorum-regulated phenotypes because they interfere with communication and compounds that simply reduce virulence factor production because they impair growth, metabolism, or cell viability (Miller & Gilmore, 2020; Lu et al., 2022; Touati et al., 2025).

7.1 Biosensor strains for quorum sensing detection

Biosensor strains are genetically or naturally responsive bacterial systems that detect quorum-sensing molecules through specific regulatory circuits and convert this detection into an observable signal. In Gram-negative bacteria, many biosensors rely on the recognition of N-acyl homoserine lactones, which are among the most widely studied autoinducers involved in LuxI/LuxR-type quorum sensing systems. These biosensors are particularly useful because AHLs differ in acyl chain length, substitution pattern, and receptor specificity, allowing the construction or selection of strains with different detection ranges and sensitivities. However, this diversity also creates methodological limitations: a biosensor that responds strongly to short-chain AHLs may be less sensitive to long-chain or 3-oxo-substituted AHLs, and a negative response in one biosensor system should not automatically be interpreted as the absence of quorum-sensing activity (Kumar et al., 2022; Miller & Gilmore, 2020).

The use of biosensor strains is especially relevant in anti-quorum sensing studies because they allow researchers to evaluate two different but related phenomena. First, they can be used to detect whether a pathogenic strain produces quorum-sensing molecules under defined culture conditions. Second, they can be used to determine whether a bioactive compound reduces the availability, perception, or biological activity of these molecules. For example, a compound may reduce biosensor activation by degrading AHLs, inhibiting AHL synthesis, blocking receptor binding, interfering with signal diffusion, or altering downstream transcriptional responses. Therefore, biosensor results must be interpreted as functional evidence of altered quorum-sensing signaling rather than as direct proof of one single mechanism unless they are combined with molecular, genetic, or chemical analyses (Miller & Gilmore, 2020; Kumar et al., 2022; Hu et al., 2024).

Whole-cell biosensors are particularly attractive because they preserve a biologically responsive environment in which signal recognition, regulatory activation, and reporter output occur within a living cell. Their advantages include relative simplicity, low cost, compatibility with microplate formats, and suitability for preliminary screening of complex natural extracts or large compound libraries. Recent developments in synthetic biology have further expanded their potential by enabling the rational design of sensing modules, genetic circuits, reporter outputs, and signal amplification strategies. Nevertheless, whole-cell biosensors remain sensitive to confounding factors such as compound cytotoxicity, medium composition, pH changes, solvent effects, pigment interference, fluorescence quenching, and metabolic burden imposed by reporter constructs (Chen et al., 2023; Nemer et al., 2024; Miller & Gilmore, 2020).

Cell-free biosensing approaches offer another methodological direction by removing some constraints associated with living reporter strains. These systems can be designed to detect quorum-sensing molecules through synthetic transcriptional or translational modules, and they may reduce risks related to biosafety, cellular toxicity, or strain instability. Their usefulness is

particularly evident when testing compounds that are strongly antimicrobial, highly colored, or chemically reactive, because such compounds may compromise living biosensor viability or distort reporter output. However, cell-free systems may not fully reproduce the physiological context of bacterial signal perception, membrane transport, intracellular metabolism, and transcriptional regulation. For this reason, cell-free biosensors should be considered complementary to, rather than replacements for, whole-cell reporter systems (Miller & Gilmore, 2020; Green et al., 2025).

7.1.1 *Chromobacterium violaceum* CV026

Among the most widely used quorum-sensing biosensors, *Chromobacterium violaceum* CV026 occupies a central place in preliminary screening studies. This mutant strain does not produce its own short-chain AHL signal but retains the ability to respond to exogenous AHLs by producing violacein, a purple pigment whose presence or inhibition can be visually observed and quantitatively measured. Because of this property, CV026 has been extensively used to detect AHL-producing bacteria and to screen potential quorum-sensing inhibitors from plant extracts, essential oils, nanoparticles, microbial metabolites, and purified bioactive compounds (Dimitrova & Kaloyanova, 2023; Miller & Gilmore, 2020).

In anti-quorum sensing studies, CV026 is commonly used in two related formats. In signal detection assays, the biosensor is exposed to supernatants or extracts from suspected AHL-producing bacteria, and violacein production indicates the presence of compatible signaling molecules. In inhibition assays, the biosensor is exposed to a known AHL source together with the test compound, and a reduction in violacein production suggests interference with AHL-mediated activation. This model has been useful for evaluating plant-derived compounds and essential oils, including studies using *Chromobacterium violaceum* and *Pseudomonas aeruginosa* as complementary models for anti-quorum sensing activity (Noumi et al., 2018). More recent applications have also used pigment-based and virulence-associated readouts to examine anti-quorum sensing effects of green-synthesized nanoparticles and plant-mediated nanomaterials (Qais et al., 2020; Qais et al., 2021; Rather & Mandal, 2023).

The principal strength of CV026 lies in its simplicity. It provides a rapid, inexpensive, and visually interpretable indication of AHL-mediated signaling. This makes it particularly useful in early-stage studies where many extracts or fractions must be screened before more detailed analyses are performed. However, its simplicity is also its main limitation. CV026 does not represent the full complexity of pathogenic quorum-sensing networks, and its response is biased toward specific AHL structures. A compound that inhibits violacein production may act on quorum sensing, but it may also interfere with pigment biosynthesis, bacterial metabolism, cell growth, membrane integrity, or the optical properties of the assay. Therefore, CV026 results should always be supported by growth controls, solvent controls, positive quorum-sensing inhibitor controls, and complementary assays in pathogenic bacteria such as *P. aeruginosa*, *Staphylococcus aureus*, *Vibrio* spp., or other clinically relevant models (Dimitrova & Kaloyanova, 2023; Miller & Gilmore, 2020; Touati et al., 2025).

When studying nanoparticles, the interpretation of CV026 assays requires additional caution. Nanoparticles can adsorb signaling molecules, scatter light, interfere with pigment extraction, alter membrane permeability, or exert mild antimicrobial effects at concentrations that appear "sub-inhibitory" in standard MIC assays. Consequently, an apparent reduction in violacein production may reflect genuine quorum-sensing interference, but it may also result from physicochemical interactions between nanoparticles, AHL molecules, or the pigment itself. For this reason, nanoparticle-based anti-quorum sensing studies should combine CV026 screening with viability assays, direct signal quantification, phenotypic assays in pathogenic strains, and, when possible, molecular or metabolomic confirmation (Qais et al., 2020; Rather & Mandal, 2023; Hu et al., 2024).

7.1.2 *Agrobacterium tumefaciens* biosensors

Agrobacterium tumefaciens biosensors represent another important group of reporter systems for detecting AHL-mediated quorum sensing. These biosensors are generally engineered to respond to a broad spectrum of AHL molecules through reporter constructs that generate colorimetric, fluorescent, or luminescent outputs. Compared with CV026, some *A. tumefaciens*-based biosensors may offer broader sensitivity to structurally diverse AHLs, making them useful when the identity of the signaling molecules produced by a test organism is unknown. They are therefore particularly valuable for environmental, clinical, and mixed-sample screening in which multiple AHLs may be present simultaneously (Miller & Gilmore, 2020; Kumar et al., 2022).

The methodological strength of *A. tumefaciens* biosensors lies in their compatibility with quantitative and high-throughput formats. Recent work has demonstrated that bacterial biosensors based on *A. tumefaciens* can be adapted for sensitive and quantitative screening of quorum-sensing inhibitors, allowing the evaluation of compound libraries under standardized microplate conditions (Zhang et al., 2023). This is especially important for anti-quorum sensing research because many candidate compounds show weak or moderate activity that may be missed by purely visual assays. Quantitative biosensor

systems can detect partial inhibition, concentration-dependent responses, and differences in activity between structurally related compounds (Zhang et al., 2023; Lu et al., 2022).

Nevertheless, *A. tumefaciens* biosensors are not free from interpretative constraints. Reporter activation depends not only on the presence of quorum-sensing molecules but also on signal uptake, receptor affinity, promoter architecture, cellular physiology, and assay conditions. Inhibition of reporter output may reflect direct antagonism of the sensing pathway, but it may also result from toxicity toward the biosensor strain or interference with reporter expression. Therefore, experiments using *A. tumefaciens* biosensors should include biosensor growth monitoring, known AHL standards, appropriate positive and negative controls, and ideally parallel detection by chromatographic or mass spectrometry-based methods when mechanistic claims are made (Miller & Gilmore, 2020; Zhang et al., 2023; Green et al., 2025).

7.1.3 Lux-based reporter systems

Lux-based reporter systems provide a powerful approach for studying quorum sensing because they convert regulatory activation into bioluminescence, which can be measured continuously, non-destructively, and with high temporal resolution. In these systems, quorum-sensing promoters or regulatory elements are linked to luciferase-based reporters, allowing researchers to follow the dynamics of signal-dependent gene expression over time. This is particularly useful when evaluating compounds that may delay, reduce, or destabilize quorum-sensing activation rather than completely abolish it (Miller & Gilmore, 2020; Chen et al., 2023).

Luminescent reporter systems offer several advantages over pigment-based assays. They are highly sensitive, compatible with real-time monitoring, and suitable for kinetic analyses. They can reveal whether a compound affects the onset of quorum sensing, the amplitude of reporter expression, the duration of activation, or the stability of the quorum-sensing response. This temporal dimension is important because quorum sensing is not a static phenomenon; it depends on population density, nutrient status, growth phase, signal accumulation, and regulatory feedback. Recent single-cell and population-level analyses of LasR-mediated quorum-sensing responses in *P. aeruginosa* illustrate the importance of resolving quorum-sensing behavior beyond endpoint measurements, particularly when linking signaling activity to bacterial growth and phenotypic heterogeneity (Ábrahám et al., 2024).

Lux-based systems are also useful for engineered pathogen-detection platforms. For example, whole-cell biosensors based on quorum-sensing recognition modules have been developed for point-of-care detection of waterborne bacterial pathogens such as *P. aeruginosa* and *Burkholderia pseudomallei*, using pathogen-associated quorum-sensing signals as diagnostic cues (Wu et al., 2021). Such systems illustrate how quorum-sensing biosensors can move beyond basic laboratory screening toward applied diagnostic and environmental monitoring contexts. However, their use in anti-quorum sensing studies still requires careful discrimination between true inhibition of signaling and nonspecific reduction of luminescence caused by impaired metabolism, reduced ATP availability, oxygen limitation, compound autofluorescence or optical interference, and reporter instability (Wu et al., 2021; Miller & Gilmore, 2020; Chen et al., 2023).

Lux-based reporters can also be integrated into high-throughput formats, making them suitable for screening large numbers of bioactive compounds. Their sensitivity allows detection of subtle quorum-sensing modulation at concentrations below the MIC, which is essential when the objective is to identify antivirulence agents rather than conventional antibiotics. However, luminescent systems should not be used as standalone proof of quorum-sensing inhibition. Ideally, reporter inhibition should be followed by phenotypic confirmation, gene expression analysis, and chemical detection of signaling molecules, particularly when the tested compounds are complex plant extracts, essential oils, or nanoparticles with multiple possible biological targets (Lu et al., 2022; Touati et al., 2025; Hu et al., 2024).

7.2 High-throughput screening methods

High-throughput screening has become increasingly important in anti-quorum sensing research because the chemical diversity of candidate compounds is expanding rapidly. Natural products, medicinal plant extracts, microbial metabolites, postbiotics, synthetic analogues, repurposed drugs, and nanoparticles can generate large experimental libraries that cannot be efficiently evaluated using only low-throughput phenotypic assays. High-throughput approaches allow researchers to screen many samples under standardized conditions, identify active candidates, establish preliminary concentration–response profiles, and prioritize compounds for deeper mechanistic analysis (Lu et al., 2022; Zhang et al., 2023; Addis et al., 2025).

In the context of quorum-sensing inhibition, high-throughput screening usually relies on microplate-compatible biosensors or reporter strains. The test compound is incubated with a biosensor and a defined quorum-sensing signal or signal-producing

strain, and the output is measured as absorbance, fluorescence, luminescence, or pigment intensity. A reliable screening workflow should simultaneously measure biosensor viability or growth because compounds that inhibit reporter output through toxicity must be excluded or interpreted separately. This is particularly important when working with crude extracts or nanoparticles, which may contain antimicrobial, redox-active, surfactant-like, or optically interfering constituents (Miller & Gilmore, 2020; Zhang et al., 2023; Addis et al., 2025).

The major advantage of high-throughput screening is its capacity to generate comparative data across large numbers of candidates. This enables ranking of compounds according to potency, selectivity, and concentration-dependent inhibition. Recent quantitative biosensor-based strategies have shown that high-throughput anti-quorum sensing screening can be designed to detect quorum-sensing inhibitors more sensitively and reproducibly than conventional visual assays (Zhang et al., 2023). However, the power of these platforms depends strongly on assay design. Poorly selected concentrations, absence of cytotoxicity controls, inappropriate solvent controls, or failure to normalize reporter output to growth can generate false-positive results and overestimate quorum-sensing inhibition (Lu et al., 2022; Miller & Gilmore, 2020).

For nanoparticles, high-throughput screening requires even stricter methodological control. Nanoparticles may affect bacterial quorum sensing by interfering with signal synthesis, secretion, accumulation, perception, or downstream response, but they may also produce apparent anti-quorum sensing effects through oxidative stress, membrane disruption, adsorption of autoinducers, or physical interference with optical detection systems. Recent reviews emphasize that nanomaterials can regulate bacterial quorum sensing through multiple mechanisms linked to both nanomaterial properties and environmental conditions (Hu et al., 2024). Therefore, nanoparticle screening should include particle characterization, aggregation assessment in the assay medium, optical interference controls, viability controls, and validation using non-optical or orthogonal detection methods when possible (Hu et al., 2024; Addis et al., 2025).

High-throughput methods should be understood as discovery tools rather than final proof of quorum-sensing inhibition. A compound identified as active in a screening assay should subsequently be evaluated through confirmatory phenotypic assays, gene expression studies, signaling molecule quantification, and, where relevant, infection models. This staged approach reduces the risk of reporting nonspecific antimicrobial or assay-interfering effects as quorum-sensing inhibition. It also allows researchers to distinguish between compounds that block signaling upstream, compounds that interfere with receptor activation, and compounds that act downstream on virulence expression without directly affecting quorum-sensing signal dynamics (Lu et al., 2022; Touati et al., 2025; Green et al., 2025).

7.3 Microfluidic platforms and lab-on-chip systems

Microfluidic and lab-on-chip platforms provide an advanced methodological framework for studying quorum sensing and quorum sensing-related virulence factors under conditions that more closely reproduce spatial organization, flow, gradients, nutrient heterogeneity, and surface-associated bacterial growth. Traditional batch cultures and static microplate biofilm assays are useful but often fail to capture the dynamic microenvironments in which quorum sensing operates during real infections or environmental colonization. In contrast, microfluidic devices allow precise control over flow rate, nutrient delivery, shear stress, chemical gradients, cell density, and spatial confinement, all of which can influence quorum-sensing activation and virulence expression (Subramanian et al., 2020; Yuan et al., 2023; Yu et al., 2022).

One of the major contributions of microfluidics to quorum-sensing research is the ability to observe bacterial behavior in real time. Biofilm development, microcolony formation, dispersal, motility, adhesion, and reporter activation can be monitored using microscopy, fluorescence imaging, or integrated sensors. This is particularly relevant for studying anti-quorum sensing compounds because their effects may vary over time and space. A compound may prevent early adhesion, delay microcolony maturation, reduce signal accumulation, promote biofilm dispersal, or alter the architecture of mature biofilms without necessarily producing a large change in total biomass. Such effects are difficult to interpret using endpoint crystal violet assays alone but can be captured more precisely in flow-cell or microfluidic systems (Yuan et al., 2023; Subramanian et al., 2020).

Microfluidic biofilm models are also valuable for testing bioactive compounds under flow conditions. In vivo, pathogenic bacteria are rarely exposed to static environments; they experience fluid movement in the urinary tract, respiratory secretions, bloodstream, wounds, catheters, and implanted devices. Flow affects nutrient availability, waste removal, signal dilution, and antimicrobial penetration. Therefore, compounds that appear active in static assays may behave differently under dynamic conditions. Microfluidic systems help address this limitation by allowing researchers to evaluate whether anti-quorum sensing activity persists when signals are continuously diluted or when biofilms are exposed to physiologically relevant shear stress (Yuan et al., 2023; Ortseifen et al., 2020; Subramanian et al., 2020).

Lab-on-chip systems can further integrate biosensing, imaging, chemical exposure, and biofilm monitoring into miniaturized platforms. This integration is particularly useful for studying quorum sensing because signaling is inherently dependent on local concentration thresholds, diffusion, spatial proximity, and microenvironmental constraints. Devices combining microfluidic culture chambers with fluorescent or luminescent reporter strains can reveal how quorum-sensing responses emerge within structured bacterial populations and how bioactive compounds modify these responses. Such platforms are increasingly relevant for studying complex biological systems, including host–microbe interactions, microbiome-related processes, and biofilm-associated infections (Yu et al., 2022; Yuan et al., 2023; Ortseifen et al., 2020).

Despite their strengths, microfluidic platforms also present important limitations. They require specialized equipment, technical expertise, careful device fabrication, and rigorous control of experimental parameters. Results may be influenced by channel geometry, surface material, flow regime, oxygen diffusion, evaporation, bubble formation, and adsorption of compounds to device surfaces. Plant extracts, essential oils, and nanoparticles may interact unpredictably with microfluidic materials, creating concentration gradients or surface deposition artifacts. Therefore, microfluidic studies should not be viewed as automatically superior to conventional assays; rather, they should be used when the research question requires spatial, temporal, or flow-based resolution that static methods cannot provide (Ortseifen et al., 2020; Yuan et al., 2023; Subramanian et al., 2020).

An optimal experimental strategy for investigating bioactive compounds against quorum sensing-related virulence factors should therefore combine biosensor screening, high-throughput quantification, phenotypic confirmation, molecular validation, chemical detection of signaling molecules, and advanced microfluidic analysis when appropriate. Biosensors provide early functional evidence of altered signaling; high-throughput platforms allow efficient identification of promising candidates; microfluidics reveals dynamic behavior under controlled microenvironmental conditions; and omics or analytical chemistry approaches clarify the underlying mechanisms. Used together, these methods can transform anti-quorum sensing research from descriptive screening into mechanistically robust evaluation of antivirulence potential (Miller & Gilmore, 2020; Lu et al., 2022; Zhang et al., 2023; Hu et al., 2024; Green et al., 2025).

Biosensor strains represent powerful tools for detecting quorum sensing signals and screening potential quorum sensing inhibitors. Their sensitivity, specificity, and ease of use have made them indispensable in contemporary anti-QS research. The most frequently employed biosensor strains and their applications are summarized in Table 5.

Table 5. Common biosensor strains used in quorum sensing studies

Biosensor strain / reporter system	Targeted QS signal or system	Reporter output	Main applications in QS and anti-QS studies	Major strengths	Main limitations	References
Chromobacterium violaceum CV026	Short- and medium-chain AHLs	Violacein production	Detection of AHL-producing bacteria; screening of anti-QS compounds; evaluation of pigment inhibition	Simple, inexpensive, rapid, visually interpretable; suitable for preliminary screening	Limited AHL spectrum; violacein inhibition may reflect toxicity, pigment interference, growth reduction, or metabolic stress rather than true QS inhibition	McClean et al., 1997; Blosser & Gray, 2000; Noumi et al., 2018; Miller & Gilmore, 2020; Dimitrova & Kaloyanova, 2023
Chromobacterium violaceum wild-type strains	Endogenous CviI/CviR-dependent AHL signaling	Violacein production	Evaluation of anti-QS effects on natural pigment production; complementary model for virulence-related phenotypes	Allows assessment of QS-regulated pigment production in a naturally pigmented background	Less specific than mutant biosensors; reduction in violacein must be separated from antibacterial or metabolic effects	McClean et al., 1997; Blosser & Gray, 2000; Noumi et al., 2018; Qais et al., 2020; Rather & Mandal, 2023
Agrobacterium tumefaciens AHL reporter biosensors	Broad-spectrum AHL detection	Colorimetric, fluorescent, or luminescent reporter signals	Detection of structurally diverse AHLs; screening of quorum-sensing inhibitors; quantitative microplate assays	Broader AHL sensitivity than CV026 in several formats; compatible with quantitative and high-throughput screening	Reporter response depends on signal uptake, receptor affinity, promoter architecture, biosensor physiology, and assay conditions	Miller & Gilmore, 2020; Kumar et al., 2022; Lu et al., 2022; Zhang et al., 2023; Green et al., 2025
Lux-based reporter systems	AHL-dependent LuxI/LuxR-	Bioluminescence	Real-time monitoring of QS activation; kinetic analysis of signal-	Sensitive, non-destructive, continuous, suitable	Luminescence may be altered by metabolic inhibition, ATP	Miller & Gilmore, 2020; Wu et al., 2021;

	type signaling		dependent gene expression; screening of compounds affecting onset, amplitude, or duration of QS responses	for temporal and high-throughput analyses	availability, oxygen limitation, compound optical interference, or reporter instability	Chen et al., 2023; Ábrahám et al., 2024
Vibrio harveyi AI-2 / luminescence biosensors	AI-2-mediated interspecies signaling	Bioluminescence	Detection of AI-2-like activity; evaluation of interspecies communication; testing compounds affecting LuxS/AI-2-related pathways	Useful for studying polymicrobial communication and AI-2-associated signaling	LuxS is linked to metabolism; reduced luminescence may reflect metabolic disturbance rather than specific AI-2 inhibition	Chu et al., 2024; Juszczuk-Kubiak, 2024; Cui & Kim, 2024; Sajeevan et al., 2025
Pseudomonas aeruginosa QS reporter strains	Las, Rhl, and PQS systems	Fluorescent, luminescent, or promoter-based reporter signals	Monitoring QS-regulated gene expression; evaluating inhibition of las, rhl, and pqs pathways; linking reporter activity to virulence phenotypes	Highly relevant pathogenic model; allows pathway-specific analysis when reporters are well designed	QS hierarchy varies with strain background, culture conditions, growth phase, nutrients, and clinical adaptation	Miranda et al., 2022; Zhang et al., 2025; Ábrahám et al., 2024; Wen et al., 2024
Staphylococcus aureus agr reporter strains	AgrC/AgrA/RNAIII peptide-based QS system	Fluorescent, luminescent, or promoter-based reporter signals	Evaluation of agr inhibition; analysis of toxin regulation, hemolysis, δ-hemolysin activity, RNAIII expression, and virulence attenuation	Clinically relevant Gram-positive QS model; useful for studying AIP-mediated virulence regulation	agr activity is strain-dependent; inhibition of hemolysis or toxin production may also reflect growth inhibition, toxin neutralization, or stress effects	Yamazaki et al., 2024; Williams, 2025; Podkowik et al., 2024
Enterococcus faecalis QS-related reporter or phenotype-linked systems	Peptide-mediated QS and virulence-associated regulation	Reporter signal, gelatinase activity, or virulence-associated readouts	Evaluation of biofilm formation, gelatinase activity, aggregation, adhesion, and virulence gene regulation	Relevant for persistent biofilms, endodontic infections, and device-associated infections	Reduced gelatinase or biofilm formation may reflect antimicrobial activity or metabolic stress rather than specific QS disruption	Yang et al., 2024
Streptococcus mutans competence/biofilm-associated reporter systems	Peptide-based QS linked to competence, biofilm formation, and cariogenicity	Reporter signal or biofilm-associated readouts	Evaluation of anti-QS effects in oral biofilm models; analysis of competence, acid tolerance, bacteriocin production, and dental biofilm regulation	Highly relevant for dental and oral microbiology; suitable for mono- and multispecies biofilm studies	Oral QS is strongly affected by pH, nutrients, salivary components, oxygen gradients, and polymicrobial interactions	Gao et al., 2024; Cui & Kim, 2024
Escherichia coli AI-2 / SdiA-related reporter systems	AI-2/LuxS signaling and AHL sensing through SdiA	Fluorescent, luminescent, or promoter-based reporter signals	Evaluation of AI-2-related communication, SdiA-mediated AHL detection, and interspecies signaling in Enterobacteriaceae	Useful for studying QS-like regulation in bacteria lacking classical LuxI-dependent AHL production	QS-like responses may overlap with metabolism, stress adaptation, indole signaling, and general regulatory pathways	Chu et al., 2024; Juszczuk-Kubiak, 2024; Cui & Kim, 2024; Xu et al., 2023

8. Advanced Imaging Techniques for Anti-QS Studies

Advanced imaging techniques occupy a central position in anti-quorum sensing studies because many QS-regulated phenotypes are spatially organized rather than merely biochemical. Biofilm formation, matrix accumulation, cell clustering, surface colonization, microcolony maturation, extracellular polymeric substance distribution, and cell viability gradients cannot be fully interpreted from bulk absorbance or colorimetric assays alone. In this context, microscopy provides structural and functional evidence showing whether a bioactive compound truly interferes with QS-associated community behavior or only reduces total biomass through nonspecific growth inhibition. Therefore, imaging should not be considered a decorative confirmation of phenotypic assays, but rather a complementary methodological layer that links anti-QS activity to visible changes in bacterial architecture, biofilm organization, matrix integrity, surface attachment, and cell survival patterns (Haval et al., 2025).

In studies evaluating antibacterial compounds, plant-derived metabolites, nanoparticles, peptides, or other antivirulence agents, imaging is particularly important because anti-QS activity is expected to attenuate virulence without necessarily killing the bacterial population. A compound that reduces crystal violet staining, for example, may inhibit initial adhesion, interfere with matrix synthesis, disperse mature biofilm, or simply reduce viable cell numbers. Microscopic visualization helps distinguish

these scenarios by revealing whether treated cells remain attached but poorly organized, whether the matrix becomes fragmented, whether microcolonies collapse, or whether the treatment produces direct membrane damage. Several recent anti-QS and antibiofilm investigations have therefore combined classical virulence assays with microscopy-based confirmation, especially when testing nanoparticles or natural products against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and other clinically relevant biofilm-forming pathogens (Ali et al., 2020; Maruthupandy et al., 2020; Chen et al., 2023; Owrang et al., 2024; Abdel-Fatah et al., 2024; Alenazi et al., 2024).

8.1 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy is one of the most informative imaging approaches for anti-QS studies because it allows three-dimensional visualization of hydrated biofilms with limited disruption of their native architecture. Unlike conventional light microscopy, which mainly provides two-dimensional surface information, CLSM generates optical sections across the biofilm depth and can reconstruct the spatial organization of microbial communities. This is particularly relevant for QS-related phenotypes because quorum sensing regulates collective traits such as biofilm maturation, extracellular matrix production, microcolony formation, and spatial heterogeneity. When a compound is proposed as an anti-QS agent, CLSM can demonstrate whether the treatment reduces biofilm thickness, alters microcolony architecture, decreases surface coverage, weakens structural compactness, or disrupts matrix-associated organization (Reichhardt & Parsek, 2019).

In *P. aeruginosa*, CLSM is especially useful because QS networks are strongly connected to biofilm architecture, extracellular matrix production, pyocyanin synthesis, rhamnolipid production, motility, and virulence-associated community behavior. CLSM can be combined with fluorescent stains or genetically encoded reporters to visualize bacterial cells, extracellular polysaccharides, extracellular DNA, proteins, or QS-regulated reporter activity. This makes it possible to move beyond a simple "biofilm inhibition percentage" and describe how the treated biofilm differs structurally from the untreated control. For example, an effective anti-QS compound may generate thinner biofilms, fewer mushroom-like structures, reduced microcolony density, lower matrix signal, or a more dispersed bacterial distribution without necessarily producing massive cell death. Such observations are highly valuable because they support an antivirulence interpretation rather than a purely bactericidal one (Reichhardt & Parsek, 2019; Chen et al., 2023).

A major strength of CLSM is that it can be coupled with quantitative image analysis. Modern biofilm studies increasingly rely on computational tools to extract measurable parameters from confocal image stacks, including biomass, average thickness, maximum thickness, surface coverage, roughness coefficient, biovolume, porosity, cell cluster size, spatial heterogeneity, and fluorescence intensity distribution. BiofilmQ, for instance, was developed to quantify spatial organization in microbial communities and provides a framework for analyzing biofilm architecture at single-cell and population levels. This type of analysis is particularly useful in anti-QS studies because subtle architectural modifications may be missed by endpoint biomass assays but detected through quantitative imaging (Hartmann et al., 2021).

However, CLSM also requires methodological caution. The reliability of CLSM-based conclusions depends on standardized biofilm growth conditions, consistent staining protocols, appropriate excitation and emission settings, adequate z-stack acquisition, avoidance of signal saturation, and rigorous image-processing parameters. Differences in objective lens, voxel size, laser intensity, thresholding strategy, and software selection can significantly influence quantitative outputs. For this reason, CLSM images should not be interpreted only visually; they should be accompanied by transparent acquisition settings and quantitative analysis whenever possible. Reviews focused on confocal biofilm image analysis emphasize that tool selection, segmentation strategy, image quality, and acquisition parameters must be carefully reported to improve reproducibility across studies (Mhade et al., 2023).

8.2 Scanning electron microscopy (SEM)

Scanning electron microscopy is widely used in anti-QS and antibiofilm studies because it provides high-resolution information on bacterial surface attachment, biofilm morphology, cell aggregation, matrix coverage, and surface-associated structural damage. SEM is particularly useful when the objective is to demonstrate how a bioactive compound modifies the external organization of bacterial communities on abiotic surfaces such as glass, polystyrene, stainless steel, catheter materials, membranes, or wound dressing substrates. In anti-QS studies, SEM can show whether treated bacteria form sparse, poorly organized, or disrupted biofilms compared with the dense and matrix-rich structures usually observed in untreated controls (Maruthupandy et al., 2020; Kozlov et al., 2023).

SEM is especially relevant for nanoparticle-based anti-QS investigations because nanoparticles may interact physically with bacterial envelopes, biofilm matrices, and extracellular polymeric substances. When used alongside phenotypic assays, SEM can reveal whether nanoparticles reduce biofilm density, deform bacterial cells, interfere with cell-to-cell aggregation, damage extracellular matrix continuity, or prevent the formation of mature biofilm structures. In studies using biogenic gold nanoparticles or nickel oxide nanoparticles against *P. aeruginosa*, microscopy has been used to support antibiofilm conclusions by showing structural alterations in treated biofilms and changes in bacterial surface organization (Ali et al., 2020; Maruthupandy et al., 2020).

The main advantage of SEM is its capacity to provide detailed surface morphology. It can reveal collapsed biofilm structures, reduced matrix-like material, altered cell shape, membrane irregularities, surface roughening, and decreased bacterial accumulation. These observations are particularly useful when the tested compound is suspected to interfere with adhesion, early biofilm development, or matrix stabilization. For plant-derived molecules and combined antimicrobial strategies, SEM can provide visible confirmation that a reduction in virulence-associated phenotypes corresponds to a real structural weakening of the bacterial community rather than only a spectrophotometric change (Chen et al., 2023; Owrang et al., 2024).

Nevertheless, SEM has limitations that must be explicitly acknowledged. Conventional SEM requires fixation, dehydration, drying, and coating steps, which may introduce artifacts such as biofilm shrinkage, matrix collapse, or distortion of extracellular polymeric substances. Therefore, SEM images should be interpreted as high-resolution structural evidence rather than as a fully native representation of hydrated biofilms. In anti-QS research, SEM is strongest when it is used together with CLSM, viability assays, biochemical measurements, and gene expression analysis. This combined interpretation prevents overestimating morphological alterations as direct QS inhibition when they may result from sample processing or nonspecific toxicity (Kozlov et al., 2023; Haval et al., 2025).

Recent developments have also improved the analytical value of electron microscopy in biofilm research. Digital image analysis and computational processing can support large-scale comparisons of biofilm growth, surface colonization, and biocide effects from electron microscopy datasets. Such approaches are important for anti-QS studies because they reduce reliance on subjective image selection and allow treated and untreated biofilms to be compared more systematically (Kozlov et al., 2023).

8.3 Transmission electron microscopy (TEM)

Transmission electron microscopy provides ultrastructural information that is not accessible through CLSM or SEM. While SEM mainly reveals surface morphology, TEM allows visualization of internal cellular damage, envelope disruption, intracellular electron density changes, nanoparticle localization, and alterations in bacterial ultrastructure. In anti-QS studies, TEM is particularly useful when the tested compound is a nanoparticle, a membrane-active molecule, or a bioactive agent suspected to interfere with bacterial integrity in addition to QS-regulated behavior. It can help determine whether reduced virulence factor production is associated with non-lethal antivirulence activity or with direct cellular injury (Ali et al., 2020; Owrang et al., 2024).

For nanoparticle-based studies, TEM can serve two complementary purposes. First, it can be used to characterize the nanoparticles themselves, including size, morphology, dispersion, and aggregation state. Second, it can be used to observe nanoparticle–bacterium interactions, such as particle adhesion to the cell surface, penetration into bacterial cells, envelope deformation, or intracellular accumulation. This distinction is important because claims about anti-QS activity must be separated from broader nanoparticle toxicity. If TEM shows extensive cell lysis or severe membrane destruction at the tested concentration, the observed reduction in QS-regulated phenotypes may reflect antibacterial damage rather than specific quorum sensing attenuation (Ali et al., 2020; Abdel-Fatah et al., 2024).

TEM can also strengthen mechanistic interpretation when combined with sub-MIC experimental design. In anti-QS assays, compounds are generally tested at concentrations that do not significantly inhibit bacterial growth. Under these conditions, TEM can help confirm whether treated cells retain general structural integrity while showing reduced biofilm formation or virulence factor expression. Such evidence supports the argument that the compound affects regulatory or community-level processes rather than simply killing the bacteria. Conversely, if ultrastructural damage is evident even at sub-MIC concentrations, the study should interpret anti-QS activity with caution and discuss possible overlap between antivirulence and stress-induced antibacterial mechanisms (Owrang et al., 2024; Haval et al., 2025).

The main limitations of TEM include laborious sample preparation, small field of view, potential preparation artifacts, and limited representativeness of complex biofilm communities. Because only thin sections or selected bacterial cells are examined, TEM should not be used alone to generalize biofilm-level effects. Its best methodological role in anti-QS research is to provide ultrastructural support for mechanisms suggested by phenotypic assays, molecular analyses, and broader imaging approaches such as CLSM or SEM (Ali et al., 2020; Haval et al., 2025).

8.4 Atomic force microscopy (AFM)

Atomic force microscopy offers a different type of information from optical and electron microscopy because it can measure surface topography and mechanical properties at nanoscale resolution. In biofilm and anti-QS studies, AFM can be used to investigate bacterial cell surface roughness, adhesion forces, extracellular matrix properties, stiffness, elasticity, and nanoscale morphological changes. These parameters are relevant because QS-regulated biofilm formation depends not only on gene expression and secreted virulence factors but also on physical interactions among cells, surfaces, and extracellular polymeric substances (Wang et al., 2024).

AFM is particularly useful when the tested compound is expected to interfere with adhesion, surface colonization, matrix cohesion, or biofilm mechanical stability. A compound may reduce biofilm formation not by killing cells, but by changing cell surface properties, reducing adhesive forces, disturbing matrix organization, or weakening biofilm viscoelasticity. AFM can therefore provide mechanistic information that complements crystal violet assays, CLSM biovolume analysis, and SEM morphology. In the context of anti-QS studies, such measurements are valuable because QS-regulated extracellular products often contribute to the mechanical robustness and spatial stability of mature biofilms (Wang et al., 2024; Haval et al., 2025).

Recent advances have expanded the potential of AFM for biofilm research. Large-area automated AFM enables the acquisition of high-resolution images over broader biofilm regions, reducing one of the traditional limitations of AFM, namely the difficulty of capturing representative spatial heterogeneity across large microbial communities. Automated imaging and computational stitching can help visualize early biofilm assembly, cell distribution, and morphological variation at scales that are more compatible with biofilm biology. This is particularly important for anti-QS studies because QS interference may not affect all regions of a biofilm equally; instead, it may alter local microcolony development, cell spacing, or surface-associated organization (Millan-Solsona et al., 2025).

Despite these strengths, AFM remains technically demanding. Sample preparation, substrate selection, hydration conditions, probe characteristics, scanning mode, and force settings can influence the results. AFM data are also often limited to selected areas and require careful interpretation when extrapolated to whole-biofilm behavior. Therefore, AFM should be used as a high-resolution mechanophysical complement rather than as a standalone anti-QS screening method. Its strongest contribution is obtained when nanoscale changes in adhesion, topography, or stiffness are interpreted together with molecular evidence of QS modulation and phenotypic evidence of reduced virulence factor production (Wang et al., 2024; Millan-Solsona et al., 2025).

8.5 Live/dead staining approaches

Live/dead staining is frequently used in anti-QS and antibiofilm studies to distinguish whether a compound reduces virulence-related phenotypes without killing bacterial cells or whether the observed effect is mainly due to bactericidal activity. This distinction is crucial because quorum sensing inhibition is generally defined as an antivirulence strategy that attenuates pathogenic behavior while exerting limited selective pressure for resistance. In practice, live/dead staining is often combined with CLSM or fluorescence microscopy to visualize the distribution of viable and membrane-compromised cells within treated and untreated biofilms (Reichhardt & Parsek, 2019; Tchatchiashvili et al., 2025).

The classical SYTO9/propidium iodide approach remains widely used because it provides a rapid visual estimate of bacterial membrane integrity. SYTO9 generally stains the total bacterial population, while propidium iodide enters cells with compromised membranes and produces a red fluorescence signal. In anti-QS studies, this method helps determine whether reduced biofilm thickness, altered architecture, or diminished virulence factor production occurs in biofilms that remain largely viable. If the treated biofilm shows a major increase in dead cells, the compound may be better interpreted as antibacterial or antibiofilm rather than specifically anti-QS. If the biofilm architecture is disrupted while most cells remain viable, the result is more consistent with antivirulence or QS-interfering activity (Maruthupandy et al., 2020; Tchatchiashvili et al., 2025).

However, live/dead staining should not be interpreted without caution. Membrane integrity does not always perfectly correspond to viability, metabolic activity, or culturability. Biofilm matrices, extracellular DNA, dye penetration, cell density,

and staining conditions can influence fluorescence signals. Recent work has highlighted that alternative staining approaches may improve viability assessment in bacterial biofilms and that metabolic-based probes can provide useful advantages over traditional membrane integrity assays. Therefore, anti-QS studies should avoid presenting live/dead images as absolute proof of viability unless they are supported by colony counts, metabolic assays, or additional viability measurements (Tchatchiashvili et al., 2025).

Live/dead staining is particularly powerful when integrated into a broader imaging workflow. For example, CLSM combined with live/dead staining can show whether a bioactive compound reduces biofilm biomass while preserving bacterial viability, SEM can reveal surface disruption, and molecular assays can confirm downregulation of QS-regulated genes. Such integration allows researchers to distinguish between antibiofilm, antibacterial, antiadhesive, matrix-disrupting, and anti-QS effects. This is essential because many bioactive compounds, especially nanoparticles and plant-derived molecules, may exert multiple overlapping effects depending on concentration, exposure time, bacterial species, and growth conditions (Ali et al., 2020; Maruthupandy et al., 2020; Owrang et al., 2024; Haval et al., 2025).

In addition to conventional fluorescence staining, newer dual-staining or alternative visualization strategies may help improve the accessibility and interpretability of biofilm imaging. Cost-effective staining methods can be useful in laboratories that do not have routine access to advanced microscopy platforms, especially when they allow differentiation between bacterial cells and extracellular matrix material. Although such methods cannot replace CLSM for three-dimensional architecture or live/dead imaging for viability mapping, they can complement anti-QS screening by providing preliminary visualization of biofilm organization and treatment-induced disruption (Nirmala et al., 2024).

Overall, advanced imaging techniques provide critical structural, spatial, and viability-based evidence in anti-QS studies. CLSM is most appropriate for three-dimensional biofilm architecture and quantitative spatial analysis; SEM is valuable for surface morphology and biofilm disruption; TEM provides ultrastructural information, especially in nanoparticle studies; AFM contributes nanoscale topographical and mechanical data; and live/dead staining helps distinguish antivirulence effects from direct killing. The most rigorous studies will not rely on a single imaging method but will integrate microscopy with phenotypic assays, sub-MIC validation, growth controls, gene expression analysis, and appropriate quantitative image processing. This integrated approach is necessary to support strong conclusions about the effects of bioactive compounds on QS-related virulence factors in pathogenic bacteria (Hartmann et al., 2021; Mhade et al., 2023; Haval et al., 2025).

9. and *Ex Vivo* Models for Evaluating Anti-QS Activity

The evaluation of anti-quorum sensing activity cannot rely exclusively on in vitro phenotypic assays, because quorum sensing-regulated virulence is strongly influenced by host-derived factors, immune pressure, tissue architecture, nutrient gradients, oxygen availability, and polymicrobial interactions. While microplate biofilm assays, pigment inhibition tests, motility assays, and enzyme activity measurements are indispensable for early screening, they do not fully reproduce the biological complexity encountered during infection. For this reason, in vivo and ex vivo models are increasingly used to determine whether the attenuation of quorum sensing-regulated phenotypes observed in vitro can be translated into reduced pathogenicity, improved host survival, lower inflammatory damage, or enhanced antimicrobial efficacy under infection-relevant conditions (Rodríguez-Urretavizcaya et al., 2024; Naga et al., 2024).

In the context of anti-QS research, these models occupy an intermediate or advanced position in the experimental workflow. They should generally be introduced after the tested compound has demonstrated activity at sub-inhibitory concentrations, without major effects on bacterial viability or growth kinetics, and after the modulation of QS-related phenotypes or QS-regulated genes has been confirmed. This order is important because an apparent protective effect in vivo may result from direct antibacterial activity, host immunomodulation, toxicity-induced bacterial clearance, or nonspecific stress responses rather than true quorum sensing interference. A rigorous anti-QS study should therefore integrate bacterial burden, host survival, histopathology or tissue damage markers, inflammatory readouts, and, where possible, direct quantification of QS-regulated virulence factors or signaling molecules within the infection model (Han et al., 2024; Gad et al., 2024).

9.1 *Galleria mellonella* infection model

The larvae of *Galleria mellonella* have become one of the most widely used invertebrate models for studying bacterial virulence and evaluating candidate anti-infective compounds. Their popularity is explained by their low cost, simple handling, absence of complex ethical constraints compared with vertebrate models, capacity to survive at temperatures close to mammalian physiological conditions, and innate immune responses that share several functional similarities with those of higher organisms.

These features make *G. mellonella* particularly useful for preliminary in vivo validation of anti-QS compounds before moving to vertebrate models (Ménard et al., 2021; Giammarino et al., 2024).

For anti-QS studies, *G. mellonella* is especially attractive because it allows rapid assessment of whether the inhibition of virulence-associated phenotypes observed in vitro corresponds to improved host survival. In a typical experimental design, larvae are infected with a defined bacterial inoculum and then treated with sub-inhibitory concentrations of the candidate compound, either before infection, shortly after infection, or in combination with conventional antibiotics. The main endpoints usually include larval survival, melanization, motility, bacterial load, and sometimes hemocyte density or histological examination. When the compound reduces mortality without markedly reducing bacterial counts, the result may support an anti-virulence rather than a bactericidal mechanism, although this interpretation must always be supported by complementary in vitro and molecular data (Ménard et al., 2021; Ten et al., 2023).

Recent studies illustrate the relevance of *G. mellonella* for validating quorum quenching and anti-QS strategies against *Pseudomonas aeruginosa*. Lactonase-producing endophytic bacteria, for example, have been evaluated for their ability to degrade acyl-homoserine lactone signals, attenuate QS-regulated virulence, and improve larval survival during *P. aeruginosa* infection. Such studies are methodologically valuable because they connect enzymatic disruption of QS signals with phenotypic virulence reduction and host-level protection (Fawzy et al., 2025). Similarly, small molecules identified as QS inhibitors can be tested in *G. mellonella* to determine whether reductions in pyocyanin, protease activity, elastase production, motility, or biofilm formation translate into increased host survival (Liu et al., 2024; Bernabè et al., 2025).

However, the *G. mellonella* model also has limitations. It lacks an adaptive immune system, does not reproduce organ-specific infection processes, and cannot fully model the pharmacokinetic and pharmacodynamic behavior of compounds in mammals. Variability may also arise from larval age, weight, source, storage conditions, injection route, incubation temperature, and operator technique. Therefore, *G. mellonella* should not be presented as a definitive model of therapeutic efficacy, but rather as a powerful screening and early validation platform for selecting the most promising anti-QS candidates for further investigation in vertebrate or tissue-based models (Ménard et al., 2021; Giammarino et al., 2024).

9.2 Zebrafish infection model

The zebrafish model occupies an important position between invertebrate and mammalian infection systems. Zebrafish embryos and larvae are optically transparent, develop rapidly, and allow real-time visualization of bacterial dissemination, immune cell recruitment, tissue damage, and host–pathogen dynamics. These characteristics are particularly useful when the objective is to understand how anti-QS compounds affect infection progression rather than simply measuring survival as an endpoint (Basheer et al., 2020; Franza et al., 2024).

In anti-QS research, zebrafish models are particularly valuable for pathogens such as *P. aeruginosa*, whose virulence is strongly regulated by QS networks. Infection can be established by microinjection into specific anatomical sites or by immersion-based protocols, depending on the pathogen, developmental stage, and research question. The use of fluorescent bacteria or transgenic zebrafish lines enables direct monitoring of bacterial burden, neutrophil or macrophage recruitment, tissue colonization, and inflammatory responses. These advantages make zebrafish models suitable for distinguishing between compounds that simply inhibit bacterial growth and compounds that attenuate virulence expression during infection (Basheer et al., 2020; Stream et al., 2022).

A notable methodological contribution is the use of zebrafish embryo infection protocols to evaluate *P. aeruginosa* virulence and validate the in vivo efficacy of QS inhibitors. Such protocols provide a controlled system in which attenuated mutants, wild-type virulent strains, and compound-treated bacteria can be compared under standardized infection conditions. When a candidate anti-QS compound reduces embryo mortality or tissue damage while preserving bacterial viability profiles observed in vitro, it strengthens the hypothesis that virulence modulation rather than direct killing is involved (Nogaret et al., 2021).

Zebrafish models are also useful for studying complex infection contexts, including polymicrobial interactions. Since QS is not only an intra-species communication system but can also influence interspecies relationships, models involving *P. aeruginosa* and *Staphylococcus aureus* are particularly relevant. Co-infection models can reveal how QS-regulated products such as elastase, rhamnolipids, phenazines, and secreted proteases affect bacterial competition, immune stimulation, and inflammatory damage in a living host (Costes et al., 2025). This is important because compounds that show anti-QS activity against a single species in vitro may behave differently in polymicrobial environments, where signaling interference can modify ecological interactions between pathogens.

Despite these strengths, zebrafish models require careful interpretation. Embryonic and larval zebrafish do not fully reproduce adult mammalian immunity, drug metabolism, or organ-specific pathophysiology. Compound solubility, embryo permeability, pigmentation, developmental toxicity, and exposure route may influence results. Therefore, anti-QS experiments in zebrafish should include toxicity controls, non-infected treated controls, bacterial load quantification, and, where possible, imaging-based confirmation of reduced tissue invasion or inflammation. Zebrafish should be considered a strong mechanistic and intermediate validation model, particularly when combined with molecular and phenotypic confirmation of QS interference (Franza et al., 2024; Stream et al., 2022).

9.3 Murine infection models

Murine models remain among the most informative systems for evaluating anti-QS compounds under mammalian physiological conditions. Their major advantage lies in the possibility of studying infection in anatomically and immunologically relevant tissues, including the lung, wound, urinary tract, bloodstream, or implanted-device environments. In anti-QS studies, murine models are particularly useful when the candidate compound has already shown reproducible activity in vitro and in simpler in vivo systems, and when the objective is to assess therapeutic relevance, inflammatory modulation, tissue protection, and compatibility with conventional antibiotics (Rodríguez-Urretavizcaya et al., 2024; Naga et al., 2024).

For *P. aeruginosa*, murine lung infection models are especially relevant because QS controls several virulence determinants involved in respiratory pathology, including pyocyanin, elastase, proteases, rhamnolipids, biofilm development, and inflammatory injury. Recent work on palmitoleic acid showed that an anti-QS candidate can be evaluated not only by measuring QS signal molecules and QS-regulated gene expression, but also by assessing protection against lung injury in a mouse infection model. This type of design is methodologically strong because it connects molecular QS inhibition with reduction of virulence-associated pathology in a mammalian host (Han et al., 2024).

Drug repurposing studies also illustrate the value of murine models for anti-QS validation. Miconazole and phenothiazine, for example, have been investigated for their ability to reduce QS-regulated virulence traits in *P. aeruginosa*, including biofilm formation, pyocyanin, protease, rhamnolipid, and hemolysin production, with additional assessment of QS gene expression. When such phenotypic and transcriptional effects are further connected to reduced pathogenicity in animal models, they provide a stronger argument for anti-virulence activity than in vitro assays alone (Gad et al., 2024).

Nevertheless, murine models must be used with methodological caution. A decrease in bacterial load in infected tissues may reflect antibacterial activity, immune stimulation, altered host physiology, or improved antibiotic penetration rather than direct QS inhibition. Conversely, a compound may reduce inflammation or tissue damage without reducing bacterial burden, which may be consistent with anti-virulence activity but still requires confirmation through QS-specific biomarkers. Therefore, the most robust murine anti-QS studies should include untreated infected controls, vehicle controls, antibiotic controls, compound-only toxicity controls, bacterial burden quantification, histopathology, cytokine analysis, and targeted measurement of QS-regulated virulence factors or gene expression in recovered bacteria or infected tissues (Han et al., 2024; Gad et al., 2024).

Murine models are also limited by cost, ethical constraints, inter-animal variability, and differences between mouse and human infection biology. These limitations are particularly important for chronic biofilm-associated infections, where standard acute infection models may fail to reproduce long-term bacterial adaptation, matrix development, tissue remodeling, and persistent inflammatory responses. For this reason, murine studies should be interpreted as part of a broader validation pipeline rather than as isolated proof of anti-QS efficacy (Rodríguez-Urretavizcaya et al., 2024; Naga et al., 2024).

9.4 Organotypic and 3D biofilm models

Organotypic and three-dimensional infection models provide an important bridge between conventional in vitro assays and animal studies. Unlike static abiotic biofilm assays, these systems incorporate host cells, extracellular matrix components, tissue-like architecture, differentiated epithelial barriers, mucus production, oxygen gradients, and more realistic nutrient conditions. This makes them particularly useful for studying QS-regulated virulence in biofilm-associated infections, where bacterial behavior is shaped by surface attachment, host-derived signals, spatial structure, and local microenvironmental heterogeneity (Crabbé et al., 2017; Grassi et al., 2024).

In respiratory infection research, 3D lung epithelial models have been used to study *P. aeruginosa* biofilm formation and antimicrobial efficacy on biotic surfaces. These models are relevant for anti-QS studies because they allow researchers to determine whether a candidate compound can reduce virulence expression or biofilm-associated tolerance in a host-cell context rather than on plastic alone. Compared with standard microplate assays, 3D models can reveal whether the presence of

epithelial cells modifies bacterial susceptibility, biofilm architecture, or the apparent efficacy of anti-virulence compounds (Crabbé et al., 2017).

Recent advances have further improved the physiological relevance of these models. Optimized alveolar epithelial cell systems have been developed to study chronic *P. aeruginosa* and *S. aureus* infections, including coinfection contexts that are highly relevant for chronic respiratory diseases. These models are particularly valuable because QS-regulated products from *P. aeruginosa* can influence not only its own virulence but also interspecies competition and host inflammatory responses (Admella et al., 2025). In such systems, anti-QS compounds can be evaluated by measuring biofilm biomass, bacterial viability, epithelial integrity, cytokine production, tissue damage markers, and pathogen-specific virulence factors.

Organotypic models are also useful for investigating specific QS-regulated virulence factors under conditions that more closely resemble infection. For example, elastase B produced by cystic fibrosis isolates of *P. aeruginosa* has been studied in organotypic 3D lung epithelial models to better understand its role in modulating host immune responses. This type of approach is highly relevant for anti-QS research because LasB is regulated by QS networks and contributes to tissue damage, immune evasion, and chronic infection phenotypes (Wauters et al., 2025).

The main limitation of organotypic and 3D models is their technical complexity. They require specialized culture conditions, longer preparation time, careful standardization, and more sophisticated endpoints than conventional in vitro assays. Reproducibility may be affected by cell source, differentiation status, matrix composition, infection dose, incubation time, and model architecture. Nevertheless, these models provide a more biologically meaningful platform for determining whether anti-QS compounds remain active in the presence of host tissues and complex infection microenvironments (Grassi et al., 2024; Admella et al., 2025).

9.5 Organ-on-chip systems

Organ-on-chip systems represent one of the most advanced methodological platforms for studying host–pathogen interactions and evaluating anti-QS strategies under dynamic, tissue-like conditions. These microfluidic devices can reproduce key physiological parameters such as fluid flow, shear stress, air–liquid interface, epithelial barrier organization, mechanical stimulation, vascular-like perfusion, and compartmentalized host–microbe interactions. Such features are particularly relevant for anti-QS studies because QS-regulated phenotypes are sensitive to spatial structure, nutrient gradients, signal diffusion, and local population density (Yokoi et al., 2023; Alonso-Roman et al., 2024).

For bacterial infection research, organ-on-chip models offer several advantages over static culture systems. They allow real-time observation of infection dynamics, controlled exposure to compounds, continuous nutrient supply, removal of planktonic cells or secreted products, and integration of host responses in a more physiologically realistic environment. In the case of *P. aeruginosa*, these platforms can be used to examine how anti-QS compounds affect biofilm development, epithelial damage, inflammatory signaling, and persistence under flow conditions that better resemble respiratory or vascularized tissues (Yokoi et al., 2023; Alonso-Roman et al., 2024).

Recent tissue-on-chip developments have shown that fully differentiated lung epithelia at the air–liquid interface can be colonized with *P. aeruginosa* in dynamic microfluidic systems. Such models are particularly promising for anti-QS research because they permit live imaging of bacterial infection, evaluation of toxin clearance, comparison between acute and chronic infection conditions, and testing of compounds under controlled flow. This is methodologically important because QS signals and secreted virulence factors accumulate differently under static and dynamic conditions, which may strongly influence the apparent efficacy of QS inhibitors (Fuglsang-Madsen et al., 2025).

Organ-on-chip models may also help reduce the gap between animal models and human infection biology. Because they can incorporate human cells and tissue-specific architecture, they provide opportunities to evaluate anti-QS compounds in systems that are more human-relevant than murine models while remaining experimentally controllable. This is particularly useful for chronic infections, where host tissue structure, epithelial differentiation, mucus, shear stress, and local inflammatory signaling strongly influence bacterial virulence expression (Alonso-Roman et al., 2024; Grassi et al., 2024).

However, organ-on-chip systems are not yet routine tools for anti-QS screening. Their limitations include high cost, technical expertise, limited throughput, device-to-device variability, difficulties in long-term standardization, and the need for specialized imaging and analytical workflows. In addition, the interpretation of QS inhibition in microfluidic systems requires careful consideration of signal dilution, flow-mediated removal of autoinducers, and altered bacterial density. Therefore, organ-on-chip experiments should be designed with appropriate static controls, flow controls, bacterial growth controls, and direct

measurements of QS-related outputs whenever possible (Yokoi et al., 2023; Alonso-Roman et al., 2024; Fuglsang-Madsen et al., 2025).

Taken together, in vivo and ex vivo models strengthen anti-QS research by moving beyond simple inhibition of isolated virulence phenotypes toward biologically meaningful evidence of reduced pathogenicity. *Galleria mellonella* provides a rapid and accessible host survival model; zebrafish enables imaging-based analysis of infection dynamics; murine models offer mammalian validation of tissue protection and inflammatory outcomes; organotypic and 3D systems reproduce host-cell-associated biofilm behavior; and organ-on-chip platforms introduce dynamic, human-relevant microenvironments. The most convincing studies will not rely on a single model but will combine complementary systems, ensuring that anti-QS activity is supported by phenotypic, molecular, host-response, and infection-outcome evidence.

10. In Silico and Computational Approaches

In silico and computational approaches have become an increasingly important methodological layer in anti-quorum sensing research because they allow the preliminary identification, prioritization, and mechanistic exploration of candidate quorum sensing inhibitors before extensive biological validation. In studies dealing with bioactive compounds, including plant-derived metabolites, marine natural products, antimicrobial peptides, nanoparticles-associated compounds, repurposed drugs, and synthetic analogues, computational tools can help predict whether a molecule may interact with QS-related proteins, disturb ligand–receptor recognition, alter receptor stability, interfere with DNA-binding domains, or affect broader virulence-associated regulatory networks (Fernandes et al., 2024; Alisaac, 2025; Hetta et al., 2024). These approaches are particularly useful when large chemical libraries must be screened against major QS-related targets such as LasR, RhlR, QscR, CviR, LuxR-type receptors, AgrA, PqsR/MvfR, LasI, RhlI, or other components involved in signal synthesis, signal detection, and transcriptional regulation (Siam et al., 2024; Kanak et al., 2023; Chaieb et al., 2022).

However, computational approaches should not be interpreted as direct proof of anti-QS activity. Molecular docking, molecular dynamics simulations, pharmacophore modeling, virtual screening, machine learning, and network-based analyses generate hypotheses that must be biologically confirmed. Their value lies in reducing experimental uncertainty, proposing plausible binding mechanisms, identifying promising chemical scaffolds, and guiding phenotypic, molecular, and in vivo validation (Fernandes et al., 2024; Alisaac, 2025). A compound predicted to bind strongly to LasR, CviR, PqsR, or AgrA may still fail experimentally because of poor solubility, weak permeability, metabolic instability, low intracellular availability, cytotoxicity, chemical instability in culture media, or inability to reach the relevant bacterial target under physiological conditions (Chaieb et al., 2022; Fernandes et al., 2024). Therefore, in silico data should be positioned as a mechanistic and prioritization layer rather than as a substitute for microbiological, molecular, and infection-relevant assays.

10.1 Molecular docking studies

Molecular docking is one of the most widely used computational strategies for exploring potential interactions between bioactive compounds and QS-related protein targets. It estimates the preferred orientation of a ligand within a binding pocket and provides predicted binding scores, interaction profiles, and possible residues involved in ligand recognition (Fernandes et al., 2024; Alisaac, 2025). In anti-QS studies, docking is commonly applied to transcriptional regulators, response regulators, and signal-synthesis proteins that influence virulence gene expression after interaction with cognate autoinducers or regulatory partners (Siam et al., 2024; Kanak et al., 2023; Chaieb et al., 2022).

A rigorous docking workflow should begin with biologically justified target selection. The selected protein must correspond to the pathogen, the QS system, and the virulence phenotype under investigation. LasR, RhlR, LasI, RhlI, and PqsR/MvfR are especially relevant for *Pseudomonas aeruginosa*; CviR is relevant for *Chromobacterium violaceum* and LuxR-type AHL-dependent systems; and AgrA is relevant for the peptide-mediated agr system of *Staphylococcus aureus* (Siam et al., 2024; Kanak et al., 2023; Murali et al., 2023; Yamaguchi et al., 2024). The target structure should be prepared carefully by removing irrelevant crystallographic molecules, retaining biologically meaningful cofactors or native ligands when required, assigning appropriate protonation states, checking missing residues, validating the binding pocket, and, where possible, redocking the native ligand to assess whether the protocol can reproduce a biologically plausible pose (Fernandes et al., 2024).

The main strength of molecular docking is its capacity to rapidly rank compounds and generate plausible mechanistic hypotheses. It may suggest whether a compound behaves as a competitive antagonist by occupying the autoinducer-binding pocket, whether it interacts with residues involved in ligand stabilization, or whether it may interfere with conformational transitions required for receptor activation (Siam et al., 2024; Revanasiddappa et al., 2024; Favero et al., 2023). Nevertheless,

docking has important limitations. Binding scores are approximate, receptor flexibility is often simplified, solvent effects are incompletely represented, and the best-scoring pose is not always the biologically relevant one (Alisaac, 2025; Fernandes et al., 2024). For this reason, docking results should be interpreted through the coherence of interaction patterns, consistency with known binding residues, comparison with native ligands or validated inhibitors, and subsequent molecular dynamics or experimental validation rather than by docking score alone (Chaieb et al., 2022; Singothu & Bhandari, 2025).

10.1.1 LasR targeting

LasR is one of the most intensively studied QS targets because it occupies a central position in the hierarchical QS network of *P. aeruginosa*. It responds primarily to N-3-oxo-dodecanoyl-L-homoserine lactone and regulates several virulence-associated traits, including elastase production, protease secretion, pyocyanin production, motility, biofilm maturation, and other collective pathogenic behaviors (Siam et al., 2024; Manson et al., 2024). Because of this central regulatory role, LasR is frequently used as a docking target in studies screening natural products, phytochemicals, marine metabolites, repurposed drugs, synthetic molecules, and nanoparticle-associated compounds for potential anti-QS activity (Siam et al., 2024; Singothu & Bhandari, 2025; Kanak et al., 2023).

In LasR docking studies, the most relevant question is not simply whether a molecule binds to the receptor, but whether it can occupy or perturb the ligand-binding domain in a way that prevents productive receptor activation. Some compounds may mimic the native acyl-homoserine lactone and behave as competitive antagonists, whereas others may bind to alternative regions and destabilize the active conformation of the receptor (Manson et al., 2024; Siam et al., 2024). Docking can identify hydrogen bonds, hydrophobic contacts, π–π interactions, and van der Waals interactions with residues located within or near the LasR ligand-binding pocket, thereby supporting a plausible mechanism of interference with native signal recognition (Siam et al., 2024; Singothu & Bhandari, 2025).

LasR docking becomes more convincing when it is associated with phenotypic and molecular validation. A compound that docks favorably to LasR and simultaneously reduces pyocyanin production, elastase activity, protease secretion, motility, and biofilm formation at sub-inhibitory concentrations provides a stronger anti-QS profile than a compound supported only by docking data (Manson et al., 2024; Singothu & Bhandari, 2025). Recent LasR-focused studies increasingly combine virtual screening, docking, binding free energy estimation, and molecular dynamics simulations to move beyond static ligand–receptor predictions and toward more integrated mechanistic interpretation (Siam et al., 2024; Singothu & Bhandari, 2025; Alisaac, 2025).

The major limitation of LasR targeting is that *P. aeruginosa* virulence regulation is not controlled by LasR alone. LasR interacts functionally with RhlR, PqsR/MvfR, QscR, and additional global regulatory systems, meaning that inhibition of LasR may produce partial or context-dependent effects if downstream circuits compensate for LasR disruption (Kanak et al., 2023; Alisaac, 2025). Conversely, some compounds may appear to affect LasR-regulated phenotypes but actually act through nonspecific effects on membrane integrity, bacterial metabolism, oxidative stress responses, or growth kinetics. For this reason, LasR docking should ideally be integrated with qRT-PCR analysis of *lasI/lasR*, *rhlI/rhlR*, and *pqsA/pqsR*, quantification of QS signals, growth controls, and phenotypic assays targeting multiple QS-controlled outputs (Kanak et al., 2023; Fernandes et al., 2024).

10.1.2 LuxR targeting

LuxR-type regulators constitute a broad family of QS transcription factors found in many Gram-negative bacteria. They generally respond to acyl-homoserine lactone signals and control collective behaviors such as luminescence, biofilm formation, pigment production, exoenzyme secretion, motility, plasmid transfer, and virulence expression (Murali et al., 2023; Revanasiddappa et al., 2024). Because LuxR-type regulators differ considerably across bacterial species, docking studies must be designed with species-specific precision. Docking a compound against CviR from *Chromobacterium violaceum*, TraR from *Agrobacterium tumefaciens*, QscR from *P. aeruginosa*, or another LuxR-type regulator does not automatically prove activity against all AHL-dependent QS systems (Murali et al., 2023; Favero et al., 2023; Revanasiddappa et al., 2024).

LuxR-type docking is particularly useful for compounds structurally related to AHLs or for bioactive molecules previously associated with QS interference, such as furanone-like scaffolds, lactone derivatives, phenolics, flavonoids, sulfur-containing phytochemicals, and fungal or plant metabolites (Favero et al., 2023; Murali et al., 2023; Revanasiddappa et al., 2024). These compounds may compete with native autoinducers, distort the ligand-binding pocket, or affect the conformational state required for DNA binding and transcriptional regulation. For this reason, LuxR-type docking studies often examine binding

energy, ligand orientation, hydrogen bonding, hydrophobic stabilization within the ligand-binding domain, and interactions with residues implicated in receptor activation (Murali et al., 2023; Revanasiddappa et al., 2024).

The advantage of LuxR-focused docking is that it can help explain broad-spectrum anti-QS effects when a compound inhibits AHL-dependent phenotypes in several Gram-negative bacteria. A molecule that interacts with multiple LuxR-type receptors may reduce violacein production in *C. violaceum*, inhibit reporter activation in biosensor strains, or attenuate AHL-regulated biofilm and pigment production in pathogenic bacteria (Favero et al., 2023; Murali et al., 2023). However, interpretation must remain cautious because LuxR-type proteins differ in ligand specificity, pocket architecture, receptor stability, and regulatory behavior. Therefore, a strong docking result against one LuxR-type regulator should not be generalized without pathogen-specific biological validation (Revanasiddappa et al., 2024; Fernandes et al., 2024).

A robust LuxR-targeting study should combine docking with biosensor assays, such as *C. violaceum* CV026, *Agrobacterium tumefaciens* reporter systems, or lux-based reporter systems, followed by confirmation in the target pathogen. This is important because reporter inhibition may result from toxicity, pigment interference, signal degradation, metabolic suppression, or direct inhibition of the biosensor strain rather than true LuxR antagonism (Favero et al., 2023; Fernandes et al., 2024).

10.1.3 AgrA targeting

AgrA is a central response regulator in the accessory gene regulator system of *Staphylococcus aureus*. Unlike many Gram-negative QS systems that rely on AHLs, the agr system is driven by autoinducing peptides detected by AgrC, which then activates AgrA through phosphorylation. Activated AgrA regulates transcription of RNAII and RNAIII and thereby influences virulence determinants such as hemolysins, phenol-soluble modulins, proteases, immune evasion factors, and biofilm-associated phenotypes (Ramasamy et al., 2023; Yamaguchi et al., 2024). This makes AgrA an important computational target for anti-virulence strategies against *S. aureus*, including methicillin-resistant strains (Ramasamy et al., 2023; Manu et al., 2024).

AgrA docking differs from LasR or LuxR docking because the objective is not necessarily to block a small AHL-like ligand-binding pocket. Instead, candidate molecules may be designed or screened to interfere with AgrA activation, dimerization, DNA binding, or conformational stability (Ramasamy et al., 2023; Yamaguchi et al., 2024). Some studies focus on the LytTR DNA-binding domain of AgrA, while others explore regions involved in activation or regulatory function. Docking must therefore be guided by a clear mechanistic hypothesis: whether the compound is expected to block AgrA–DNA interaction, destabilize the active conformation, or indirectly interfere with agr-mediated signal transduction (Yamaguchi et al., 2024; Manu et al., 2024).

AgrA targeting is methodologically valuable because it can connect computational predictions to measurable virulence outcomes, including reduced RNAIII expression, decreased α-hemolysin production, attenuated hemolytic activity, altered toxin regulation, or reduced host-cell damage (Yamaguchi et al., 2024). However, interpretation must be careful because agr inhibition may reduce toxin production while sometimes increasing biofilm-associated phenotypes, since agr activity is often involved in the transition from adhesive biofilm growth to dispersal and invasive virulence (Ramasamy et al., 2023; Yamaguchi et al., 2024). Therefore, an AgrA-targeting compound should not be evaluated using a single endpoint. It should be tested for toxin production, hemolysis, biofilm formation, dispersal, agr-regulated gene expression, and infection-relevant outcomes where possible.

Recent AgrA-focused studies illustrate the value of combining machine learning, pharmacophore modeling, docking, molecular dynamics simulations, and experimental validation. Such workflows are stronger than isolated docking because they assess whether the predicted ligand–protein complex remains stable over time and whether the computationally proposed mechanism corresponds to measurable reduction of agr-regulated virulence (Ramasamy et al., 2023; Yamaguchi et al., 2024; Manu et al., 2024).

10.1.4 PqsR targeting

PqsR, also known as MvfR, is a major transcriptional regulator of the quinolone signaling system in *P. aeruginosa*. It regulates the biosynthesis and response to quinolone signals such as PQS and HHQ and contributes to pyocyanin production, biofilm development, outer membrane vesicle formation, oxidative stress responses, and virulence regulation (Kanak et al., 2023; Chaieb et al., 2022). Because PqsR/MvfR is functionally connected to pathogenicity and biofilm-associated behavior, it has become a relevant target for virtual screening and structure-based anti-QS discovery (Kanak et al., 2023; Chaieb et al., 2022).

Docking against PqsR is particularly attractive because several inhibitor scaffolds can be explored in relation to quinolone-sensing mechanisms. A strong PqsR docking study should not only report binding scores but should also examine whether the compound occupies the ligand-binding pocket, interacts with residues involved in quinolone recognition, and could prevent the conformational transitions required for transcriptional activation (Kanak et al., 2023; Chaieb et al., 2022). Because PqsR-controlled phenotypes overlap with those regulated by LasR and RhlR, computational PqsR inhibition should ideally be combined with phenotypic assays for pyocyanin, biofilm formation, motility, and pqs-regulated gene expression (Kanak et al., 2023).

PqsR-focused analyses benefit from multidimensional computational workflows that include docking, molecular dynamics simulations, and binding free energy estimation. This is important because a compound may dock favorably but become unstable during simulation, or may remain stable but lack adequate physicochemical properties for biological activity (Chaieb et al., 2022; Alisaac, 2025). By integrating several computational filters, researchers can prioritize compounds that are not only predicted to interact with PqsR but also more likely to maintain stable interactions under dynamic conditions.

The limitation of PqsR targeting is that the pqs system is embedded within a broader regulatory network. Inhibiting PqsR may reduce some virulence traits while leaving others under the influence of LasR, RhlR, QscR, metabolic regulation, or stress-response pathways (Kanak et al., 2023; Alisaac, 2025). Therefore, computational PqsR inhibition should be interpreted in relation to the broader QS network and not as a standalone explanation of all anti-virulence effects.

10.2 Molecular dynamics simulations

Molecular dynamics simulations complement docking by evaluating the time-dependent behavior of ligand–protein complexes under simulated physiological conditions. Whereas docking provides a static or semi-flexible prediction of a possible binding pose, molecular dynamics explores whether this pose remains stable over time, how the protein backbone fluctuates, how the ligand behaves within the binding pocket, and whether key interactions persist or disappear during simulation (Alisaac, 2025; Singothu & Bhandari, 2025). For anti-QS studies, this is particularly important because QS regulators may undergo conformational changes that influence ligand recognition, dimerization, DNA binding, and transcriptional activation (Ramasamy et al., 2023; Yamaguchi et al., 2024).

Common molecular dynamics outputs include root mean square deviation, root mean square fluctuation, radius of gyration, solvent-accessible surface area, hydrogen bond occupancy, principal component analysis, free energy landscape analysis, and MM-PBSA or MM-GBSA binding free energy estimation (Chaieb et al., 2022; Alisaac, 2025). These parameters help determine whether a candidate inhibitor stabilizes or destabilizes the receptor, affects flexible regions, preserves key contacts, or produces a conformational profile compatible with inhibition rather than activation (Singothu & Bhandari, 2025; Yamaguchi et al., 2024).

In anti-QS research, molecular dynamics is especially useful for comparing native autoinducers, known inhibitors, and newly proposed compounds. If a candidate compound maintains stable interactions with the receptor and produces a conformational profile different from that of the native agonist, this may support an antagonist-like mechanism (Alisaac, 2025; Yamaguchi et al., 2024). Conversely, if the compound rapidly leaves the binding pocket, produces unstable interactions, or behaves similarly to an agonist, its predicted anti-QS relevance becomes weaker.

Nevertheless, molecular dynamics simulations also have limitations. Their results depend on the chosen force field, ligand parameterization, protonation states, simulation length, solvent model, ion concentration, and initial docking pose (Alisaac, 2025; Fernandes et al., 2024). Short simulations may not capture slow conformational transitions, and binding free energy calculations remain approximations. Therefore, molecular dynamics should refine hypotheses rather than declare biological activity. Its strongest use is in combination with docking, ADMET filtering, phenotypic assays, QS gene-expression analysis, and quantification of signaling molecules (Fernandes et al., 2024; Chaieb et al., 2022).

10.3 QS inhibitor prediction using artificial intelligence

Artificial intelligence and machine learning are emerging tools for predicting quorum sensing inhibitors, prioritizing chemical libraries, and identifying structural or regulatory features associated with anti-QS activity. Unlike traditional docking, which mainly relies on target structure, AI-based approaches can integrate chemical descriptors, molecular fingerprints, QS bioassay data, toxicity profiles, antimicrobial activity, gene-expression signatures, ligand–target relationships, and communication-network data (Ramasamy et al., 2023; Wu et al., 2022). This makes them attractive for large-scale screening of natural compound databases, repurposed drug libraries, marine metabolites, phytochemical collections, and synthetic analogues.

In anti-QS discovery, machine learning can be used in several ways. Classification models can predict whether a molecule is likely to behave as a QS inhibitor or non-inhibitor; regression models can estimate inhibitory potency; clustering approaches can identify chemical families enriched in anti-QS activity; and pharmacophore-assisted workflows can prioritize compounds with structural features compatible with inhibition of specific QS targets (Ramasamy et al., 2023). Machine learning can also support the construction of QS communication networks, making it possible to explore complex microbial interactions beyond single compound–single target models (Wu et al., 2022).

The main advantage of AI-based prediction is its capacity to handle large and heterogeneous datasets more efficiently than manual screening. It can also identify non-obvious chemical patterns that may not be easily recognized through classical docking or medicinal chemistry alone (Ramasamy et al., 2023; Wu et al., 2022). This is particularly relevant for distinguishing compounds that inhibit QS-related phenotypes without inhibiting bacterial growth, which is a central distinction in anti-virulence research. Many molecules reported as QS inhibitors may actually reduce virulence outputs by impairing viability, metabolism, membrane integrity, or global stress responses rather than by selectively interfering with QS regulation.

However, AI-based QS inhibitor prediction faces major methodological challenges. Available datasets remain limited, heterogeneous, and often generated using different bacterial strains, assay conditions, endpoints, concentrations, solvents, and definitions of QS inhibition (Fernandes et al., 2024; Ramasamy et al., 2023). Many published studies report inhibition of biofilm, pigment, or motility without proving that QS is the primary target. If such data are used uncritically for model training, AI models may learn general antibacterial, antibiofilm, or stress-related patterns rather than QS-specific activity. For this reason, curated datasets should distinguish growth inhibition, antibiofilm activity, anti-virulence activity, signal degradation, receptor antagonism, transcriptional QS suppression, and true quorum quenching mechanisms.

Another major challenge is interpretability. A model may predict that a compound is likely to inhibit QS, but the prediction is more useful when linked to interpretable chemical features, likely molecular targets, or experimentally testable mechanisms (Ramasamy et al., 2023). Therefore, AI prediction should be combined with docking, molecular dynamics simulations, ADMET screening, and laboratory validation. AI can prioritize candidates, but the final demonstration of anti-QS activity requires sub-MIC testing, growth controls, QS reporter assays, virulence phenotype assays, gene-expression analysis, signal quantification, and infection-relevant validation.

10.4 Network pharmacology and systems biology approaches

Network pharmacology and systems biology approaches are particularly relevant for anti-QS research because bacterial virulence is rarely governed by a single receptor or pathway. QS systems are embedded in complex regulatory networks involving metabolism, stress responses, two-component systems, cyclic-di-GMP signaling, efflux pumps, biofilm matrix production, secretion systems, iron acquisition, and host-response pathways (Wu et al., 2022; Hetta et al., 2024). A compound may therefore influence QS-related virulence either by directly targeting a QS receptor or by modulating upstream and downstream pathways that converge on virulence expression.

Network-based approaches are especially useful for complex bioactive mixtures such as plant extracts, essential oils, marine extracts, postbiotics, and nanoparticle-associated phytochemicals. These preparations often contain multiple active molecules that may act on several bacterial and host-associated targets simultaneously (Hetta et al., 2024; Fernandes et al., 2024). Rather than assuming a single compound–single target mechanism, network approaches can map compound–target–pathway relationships and identify central nodes connecting QS regulation to biofilm formation, toxin production, motility, inflammation, oxidative stress, antibiotic tolerance, and immune modulation.

In anti-QS studies, systems biology can integrate transcriptomic, proteomic, metabolomic, and phenotypic data. For example, transcriptomic analysis may reveal downregulation of *las*, *rhl*, *pqs*, *lux*, *cvi*, or *agr* regulons; proteomic analysis may show reduced abundance of secreted proteases, toxins, or biofilm-associated proteins; metabolomics may detect reduced autoinducer levels or altered virulence-associated metabolites; and phenotypic assays may confirm reduced biofilm, pigment, motility, hemolysis, or enzyme production (Hetta et al., 2024; Alisaac, 2025). When these layers converge, the evidence for QS-related anti-virulence activity becomes substantially stronger than when a study relies on a single endpoint.

The advantage of network and systems approaches is that they can help determine whether a compound produces targeted QS interference or broad physiological disruption. This distinction is essential because a compound that globally suppresses bacterial metabolism may reduce QS-regulated virulence factors without being a specific QS inhibitor (Fernandes et al., 2024; Hetta et al., 2024). Conversely, a compound that selectively downregulates QS regulons while preserving bacterial growth may

represent a more credible anti-virulence candidate. Network analysis can also reveal compensatory pathways, explain why some QS inhibitors fail in complex infection models, and support the rational design of combinations with antibiotics, quorum quenching enzymes, or other anti-virulence agents.

The main limitation of network pharmacology and systems biology is that they require high-quality datasets, rigorous bioinformatics, appropriate statistical thresholds, and cautious interpretation. Predicted networks can be biased by incomplete databases, poorly annotated bacterial targets, overrepresentation of model organisms, and assumptions transferred from human pharmacology to microbial systems (Wu et al., 2022; Fernandes et al., 2024). Therefore, computational networks should be experimentally validated through targeted gene-expression assays, mutant analysis, biochemical assays, signal quantification, and phenotypic confirmation.

Overall, in silico and computational approaches provide a powerful methodological framework for anti-QS research. Molecular docking identifies plausible receptor–ligand interactions; molecular dynamics simulations evaluate the stability and conformational behavior of these interactions; AI supports large-scale prediction and prioritization of candidate QS inhibitors; and network pharmacology or systems biology helps interpret multi-target and multi-pathway effects (Alisaac, 2025; Fernandes et al., 2024; Ramasamy et al., 2023; Wu et al., 2022). The strongest studies are those that integrate computational predictions with carefully controlled microbiological assays, QS-specific molecular readouts, and infection-relevant validation models.

11. Challenges and Limitations in Anti-Quorum Sensing Research

Despite the growing interest in quorum sensing inhibition as an anti-virulence strategy, anti-QS research still faces several methodological, biological, and translational limitations that must be carefully considered when interpreting experimental findings. Unlike conventional antibacterial studies, where growth inhibition or bacterial killing can be directly measured through standardized endpoints such as MIC, MBC, or time-kill kinetics, anti-QS investigations aim to evaluate the attenuation of bacterial communication and virulence without necessarily affecting bacterial viability (Filipić et al., 2024; Hetta et al., 2024). This distinction makes the field scientifically attractive, because it may reduce selective pressure compared with bactericidal or bacteriostatic therapies, but it also creates substantial methodological complexity, since reductions in biofilm formation, pigment production, motility, extracellular enzyme activity, or toxin secretion may result either from true quorum sensing interference or from subtle impairment of bacterial growth, metabolism, or stress tolerance (Filipić et al., 2024; Rodríguez-Urretavizcaya et al., 2024; Naga et al., 2024).

11.1 Lack of methodological standardization

One of the most persistent challenges in anti-QS research is the lack of harmonized experimental standards across laboratories. Studies frequently differ in bacterial strains, inoculum density, incubation time, culture medium, aeration conditions, compound preparation, solvent concentration, endpoint measurement, and normalization strategy, making direct comparison between results difficult (Lourenço et al., 2014; Azevedo et al., 2021; Allkja et al., 2021). This limitation is particularly evident in biofilm-related assays, where even small variations in plate material, incubation temperature, washing intensity, staining time, dye solubilization, and optical density reading can substantially affect the final quantitative output (Allkja et al., 2021; Castro et al., 2022). The MIABiE initiative was proposed to improve reporting quality in biofilm experiments by defining the minimum information required to make procedures interpretable and reproducible, but these recommendations are still not systematically adopted in many anti-QS studies (Lourenço et al., 2014; Coenye, 2023).

The absence of standardization is even more problematic when studies claim anti-QS activity on the basis of a single phenotypic endpoint. For example, inhibition of biofilm biomass measured by crystal violet staining may suggest an anti-virulence effect, but the method does not distinguish between reduced adhesion, altered matrix production, decreased bacterial viability, impaired growth, or detachment of mature biofilm structures (Castro et al., 2022; Coenye, 2023). Therefore, anti-QS conclusions should ideally be supported by multiple complementary assays, including growth controls, viability measurements, QS-regulated phenotypes, gene expression analysis, and, when possible, detection of signaling molecules (Filipić et al., 2024; Rodríguez-Urretavizcaya et al., 2024).

11.2 Distinguishing antibacterial effects from true anti-QS activity

A major conceptual limitation in the field is the frequent confusion between antibacterial activity and quorum sensing inhibition. A compound that reduces pyocyanin production, violacein synthesis, swarming motility, elastase activity, hemolysin secretion, or biofilm biomass may appear to behave as a QS inhibitor, but these phenotypes may also decrease when the compound partially inhibits bacterial growth or causes metabolic stress (Filipić et al., 2024; Hetta et al., 2024). For this

reason, anti-QS activity should be evaluated at sub-inhibitory concentrations that do not significantly affect planktonic growth, bacterial viability, or overall metabolic activity (Filipić et al., 2024; Naga et al., 2024).

The use of sub-MIC concentrations is necessary but not sufficient. Some compounds, particularly plant extracts, essential oils, phenolics, alkaloids, antimicrobial peptides, and nanoparticles, may alter membrane permeability, respiration, redox balance, or nutrient uptake at concentrations below the MIC, thereby indirectly affecting QS-regulated phenotypes without specifically targeting QS receptors, autoinducer synthesis, signal transport, or signal degradation (Filipić et al., 2024; Mondal et al., 2024; Algadi et al., 2024). This issue is especially important for complex natural extracts, because their biological effects may result from several interacting constituents rather than a single defined anti-QS molecule (Hetta et al., 2024; Naga et al., 2024). Consequently, studies should avoid interpreting phenotypic inhibition alone as proof of QS disruption unless supported by mechanistic evidence such as reduced autoinducer levels, altered expression of QS-regulated genes, receptor-binding studies, or restoration experiments using exogenous signaling molecules (Filipić et al., 2024; Rodríguez-Urretavizcaya et al., 2024).

11.3 Reproducibility issues in anti-virulence studies

Reproducibility remains a central concern in anti-virulence and anti-biofilm research. Biofilm experiments are highly sensitive to methodological details, and interlaboratory comparisons have shown that reproducibility depends strongly on experimental design, statistical treatment, and strict control of technical variables (Azevedo et al., 2021; Allkja et al., 2021). Anti-QS studies are even more vulnerable to reproducibility problems because they often combine several biologically variable endpoints, including biofilm formation, motility, pigment production, extracellular enzyme activity, and gene expression, each of which may be influenced by medium composition, oxygen availability, growth phase, population density, and strain-specific regulatory networks (Azevedo et al., 2021; Coenye, 2023).

Another source of poor reproducibility is the insufficient reporting of compound properties. Many studies do not adequately describe extract composition, nanoparticle size distribution, surface charge, synthesis method, batch variability, solvent concentration, storage conditions, or chemical stability during incubation (Algadi et al., 2024; Mondal et al., 2024; Huang et al., 2024). This is particularly problematic for nanoparticles and plant-derived products, whose physicochemical characteristics can strongly influence biological activity, toxicity, aggregation behavior, and interaction with bacterial cells or culture media (Algadi et al., 2024; Mondal et al., 2024). For anti-QS research to become more reproducible, experimental protocols should include detailed characterization of the tested compound, transparent reporting of all assay conditions, independent biological replicates, appropriate statistical models, and confirmation of key findings using more than one bacterial strain or experimental platform (Lourenço et al., 2014; Allkja et al., 2021; Filipić et al., 2024).

11.4 Variability among bacterial strains and models

The anti-QS effect of a compound may vary considerably depending on the bacterial species, strain background, QS architecture, virulence repertoire, resistance profile, and ecological context. For example, *Pseudomonas aeruginosa* possesses interconnected QS circuits, including the Las, Rhl, Pqs, and IQS systems, and inhibition of one pathway may be compensated by another regulatory network depending on strain background and environmental conditions (Rodríguez-Urretavizcaya et al., 2024). Similarly, QS-mediated regulation in *Staphylococcus aureus*, *Vibrio* spp., *Chromobacterium violaceum*, *Escherichia coli*, and polymicrobial communities does not follow a single universal model, meaning that a compound active in one biosensor system may not necessarily remain effective against clinically relevant pathogens or mixed-species biofilms (Cui et al., 2024; Hetta et al., 2024; Naga et al., 2024).

This variability is particularly important when laboratory reference strains are used as the only biological model. Reference strains are useful for methodological reproducibility and mechanistic comparison, but they may not fully reflect the phenotypic heterogeneity, adaptive resistance, virulence diversity, or biofilm-forming capacity of clinical isolates (Coenye, 2023; Vyas et al., 2022). Clinical isolates, multidrug-resistant strains, and polymicrobial models may provide more realistic information, but they also introduce greater experimental variability and require more careful interpretation (Cui et al., 2024; Cometta et al., 2024). Therefore, robust anti-QS evaluation should ideally move from simple reference-strain screening toward validation in clinically relevant strains and infection-associated models (Vyas et al., 2022; Coenye, 2023; Cometta et al., 2024).

11.5 Limitations of in vitro models

Most anti-QS studies are initially performed using static in vitro assays because these systems are simple, inexpensive, scalable, and compatible with high-throughput screening. However, static microplate assays poorly reproduce the complexity of in vivo infections, where bacteria experience nutrient gradients, host immune pressure, shear forces, tissue architecture, oxygen

limitation, antimicrobial exposure, and interactions with host cells or other microorganisms (Vyas et al., 2022; Coenye, 2023; Cometta et al., 2024). As a result, compounds that show strong anti-biofilm or anti-QS activity in microplates may fail to reproduce the same effect in flow-cell systems, ex vivo tissues, organotypic models, implanted-device models, or animal infections (Vyas et al., 2022; Cometta et al., 2024).

Another limitation is that in vitro models often simplify the biofilm into a measurable biomass endpoint, while clinically relevant biofilms are structured, heterogeneous, dynamic, and embedded in host-derived matrices. In this context, reduction of crystal violet staining does not necessarily indicate clinically meaningful biofilm control, especially when viable cells remain embedded in residual matrix or when the compound affects staining properties rather than biofilm biology itself (Castro et al., 2022; Coenye, 2023). More advanced models, including flow-based systems, 3D biofilm platforms, implant-associated biofilm models, and host-mimicking environments, may improve translational relevance, although they are technically more demanding, less standardized, and less suitable for large-scale screening (Vyas et al., 2022; Cometta et al., 2024).

11.6 Toxicological concerns associated with nanoparticles

Nanoparticles are increasingly investigated as antibacterial, antibiofilm, and anti-QS agents because of their high surface area, tunable physicochemical properties, ability to generate reactive oxygen species, interaction with bacterial membranes, and potential use as delivery systems for bioactive compounds (Algadi et al., 2024; Mondal et al., 2024; Huang et al., 2024). However, their application in anti-QS research raises important toxicological and methodological concerns. Unlike small molecules, nanoparticles may exert multiple simultaneous effects, including oxidative stress, membrane disruption, metal ion release, protein interaction, DNA damage, and interference with colorimetric or fluorometric assays (Algadi et al., 2024; Mondal et al., 2024). Therefore, a reduction in QS-related virulence factors after nanoparticle exposure may reflect broad antimicrobial stress rather than specific QS inhibition (Filipić et al., 2024; Algadi et al., 2024).

The safety profile of nanoparticles must also be evaluated carefully before considering translational applications. Parameters such as particle size, morphology, surface coating, charge, aggregation state, solubility, dose, exposure time, and route of administration can influence cytotoxicity, immunogenicity, biodistribution, persistence, and environmental impact (Mondal et al., 2024; Huang et al., 2024). This makes it necessary to include host-cell cytotoxicity assays, hemocompatibility tests, oxidative stress markers, and, where appropriate, in vivo safety evaluation alongside antibacterial and anti-QS endpoints (Algadi et al., 2024; Huang et al., 2024). Without such controls, nanoparticle-based anti-QS claims may overestimate therapeutic potential while underestimating biological risk (Mondal et al., 2024; Huang et al., 2024).

11.7 Translational challenges from laboratory to clinical application

Although QS inhibition is a promising anti-virulence strategy, its translation from laboratory experiments to clinical application remains limited. One major challenge is that reducing virulence factor expression does not always translate into improved infection outcomes, especially in established biofilms, chronic infections, immunocompromised hosts, or polymicrobial environments where bacterial survival depends on multiple redundant mechanisms (Cui et al., 2024; Coenye, 2023; Rodríguez-Urretavizcaya et al., 2024). In addition, anti-QS compounds may be more effective as adjunctive therapies than as stand-alone treatments, particularly when combined with antibiotics, wound care strategies, implant management, or immune-based interventions (Hetta et al., 2024; Rodríguez-Urretavizcaya et al., 2024; Naga et al., 2024).

Another translational barrier is the difficulty of defining clinically relevant endpoints for anti-QS agents. Conventional antimicrobial development relies on bacterial killing, growth inhibition, pharmacokinetics, pharmacodynamics, and resistance prevention, whereas anti-virulence therapies may require endpoints based on reduced tissue damage, improved immune clearance, decreased biofilm persistence, enhanced antibiotic susceptibility, or attenuation of disease severity (Filipić et al., 2024; Coenye, 2023). These endpoints are harder to standardize and may vary across infection sites, pathogens, host immune status, and treatment combinations (Vyas et al., 2022; Cometta et al., 2024).

Finally, the clinical development of anti-QS agents must address compound stability, toxicity, bioavailability, delivery to infection sites, resistance evolution, regulatory classification, and compatibility with existing antimicrobial therapy (Filipić et al., 2024; Huang et al., 2024). For nanoparticles and complex natural products, these issues are even more pronounced because composition, batch reproducibility, pharmacological behavior, and safety assessment are often more difficult to define than for single purified molecules (Mondal et al., 2024; Algadi et al., 2024; Huang et al., 2024). Therefore, the future of anti-QS research will depend not only on identifying active compounds, but also on developing rigorous methodological frameworks capable of distinguishing true QS inhibition from nonspecific antimicrobial stress, improving reproducibility, and validating promising

candidates in biologically relevant infection models (Lourenço et al., 2014; Azevedo et al., 2021; Filipić et al., 2024; Coenye, 2023).

12. Future Perspectives and Emerging Trends

The future of anti-quorum sensing research will depend on the transition from descriptive inhibition assays toward integrated methodological pipelines capable of linking compound activity, molecular targets, bacterial physiology, host relevance, and translational feasibility. Early anti-QS studies frequently relied on reductions in biofilm biomass, pigment production, motility, or extracellular enzyme activity as indicators of quorum sensing interference. Although these phenotypic endpoints remain important, they are no longer sufficient when used alone, because decreases in QS-regulated virulence factors may result from true signal interference, general metabolic stress, delayed growth, membrane damage, or nonspecific toxicity. Future studies should therefore combine phenotypic assays with MIC/sub-MIC determination, gene expression profiling, signal molecule quantification, omics approaches, imaging, and host-relevant infection models to establish whether a compound acts as a genuine QS modulator rather than as a conventional antibacterial agent with indirect antivirulence effects (Filipić et al., 2024; Mitra et al., 2024; Naga et al., 2024).

Major future directions and emerging perspectives in anti-virulence and quorum sensing inhibition research are summarized in Figure 7.

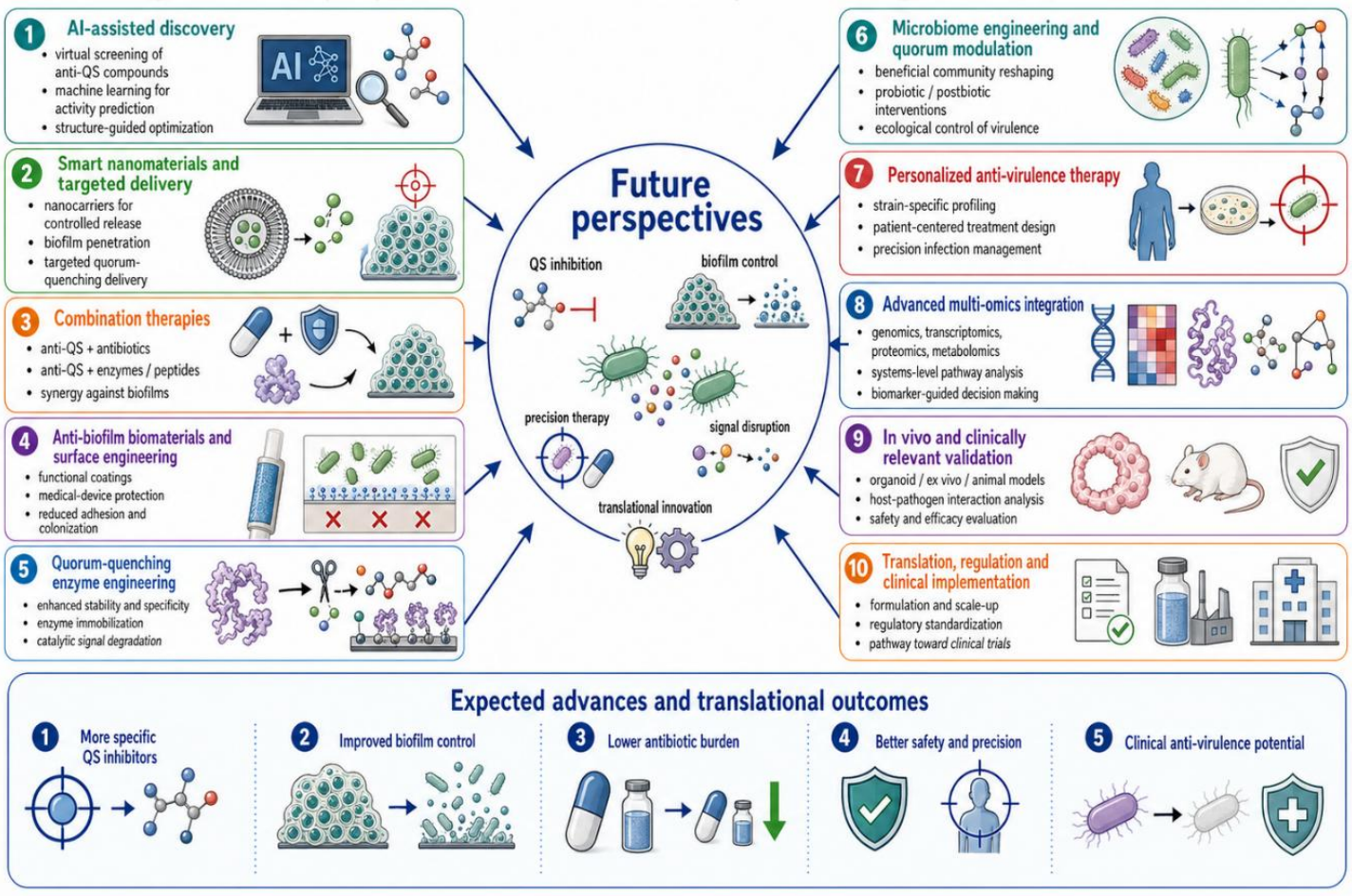

Figure 7. Future perspectives in anti-virulence and quorum sensing inhibition research

This methodological evolution is particularly important because quorum sensing does not function as an isolated regulatory pathway. It is embedded in broader networks controlling biofilm maturation, extracellular polymeric substance production, motility, toxin secretion, siderophore production, immune evasion, antibiotic tolerance, and interspecies communication. For this reason, future anti-QS research should not be limited to proving that a compound reduces one virulence phenotype; it should clarify how the intervention modifies bacterial communication, whether the effect is strain-dependent, whether it persists under infection-like conditions, and whether it improves the activity of existing antimicrobial strategies (Wang et al., 2024; Mitra et al., 2024; Naga et al., 2024).

12.1 AI-assisted discovery of anti-QS compounds

Artificial intelligence is expected to become an increasingly useful tool in the discovery of anti-QS compounds because the chemical space relevant to quorum sensing inhibition is too large to be explored efficiently by conventional screening alone. Traditional discovery approaches generally begin with crude extracts, purified phytochemicals, synthetic molecules, nanoparticles, or microbial metabolites, followed by experimental testing against QS-regulated phenotypes. This strategy remains valuable, but it is time-consuming, often poorly predictive of target specificity, and frequently limited by the absence of mechanistic validation. AI-assisted pipelines can help prioritize candidate molecules before laboratory testing by integrating chemical descriptors, receptor structures, known QSI datasets, docking scores, molecular dynamics outputs, predicted toxicity, and biological activity profiles (Almatroudi, 2025; Xu et al., 2023).

In anti-QS research, machine learning and deep learning approaches may be used to predict whether a compound is likely to interact with key regulatory proteins such as LasR, RhlR, PqsR, CviR, LuxR-type receptors, LsrK-associated AI-2 pathways, or Agr-related systems. This is particularly relevant for pathogens such as *Pseudomonas aeruginosa*, *Chromobacterium violaceum*, *Vibrio* spp., *Escherichia coli*, and *Staphylococcus aureus*, in which QS systems regulate clinically important virulence traits. Recent computational studies illustrate the value of combining virtual screening, molecular docking, molecular dynamics simulations, and biological validation to identify candidate QS inhibitors targeting LasR or AI-2-associated signaling pathways (Almatroudi, 2025; Xu et al., 2023).

However, AI should not be presented as a substitute for experimental microbiology. Its strongest value lies in reducing the number of candidates to be tested, identifying likely binding modes, predicting structure–activity relationships, and generating testable hypotheses. A robust AI-assisted anti-QS workflow should therefore begin with computational prioritization, continue with MIC and sub-MIC evaluation, proceed through phenotypic assays, confirm target-level effects through gene expression or reporter systems, and finally validate the biological relevance of the compound in biofilm, infection, or host-associated models (Filipić et al., 2024; Almatroudi, 2025).

AI-assisted discovery may also be particularly useful for natural products. Plant extracts, marine metabolites, microbial secondary metabolites, and food-derived compounds often contain complex mixtures of molecules, making it difficult to identify which constituents are responsible for anti-QS activity. Cheminformatics and AI-based dereplication can help rank candidate molecules according to their predicted affinity for QS receptors, structural similarity to autoinducers, pharmacokinetic properties, toxicity risk, and compatibility with formulation strategies. This approach is highly relevant because natural product-based QS inhibitors derived from plants, microorganisms, and marine organisms continue to represent one of the most active areas of anti-virulence research (Alum et al., 2025).

The main limitation of AI-assisted anti-QS discovery is the quality of available datasets. Many published studies use different bacterial strains, culture media, incubation times, compound concentrations, solvents, endpoints, and reporting standards. As a result, datasets are often heterogeneous and difficult to compare. Future progress will require curated anti-QS databases linking compound identity, chemical structure, bacterial species and strain, MIC, sub-MIC concentration, growth effect, QS target, phenotype, gene expression, signal molecule concentration, cytotoxicity, and in vivo outcome. Without such datasets, AI models may reproduce the biases and methodological weaknesses of the literature rather than overcome them (Filipić et al., 2024; Almatroudi, 2025).

12.2 Smart nanomaterials and targeted delivery systems

Smart nanomaterials represent one of the most promising directions in anti-QS and anti-biofilm research. Nanomaterials may influence bacterial communication at several levels, including signal synthesis, signal secretion, autoinducer accumulation, receptor interaction, signal transduction, biofilm matrix organization, and QS-regulated gene expression. Recent reviews have emphasized that nanomaterials can regulate bacterial QS through both direct and indirect mechanisms, depending on their composition, size, surface charge, coating, concentration, and interaction with the bacterial microenvironment (Hu et al., 2024).

Metal and metal oxide nanoparticles, including silver, zinc oxide, titanium dioxide, copper oxide, and iron oxide nanoparticles, have been widely investigated for their antimicrobial, antibiofilm, and antivirulence effects. Their activity may involve membrane interaction, oxidative stress, ion release, disruption of extracellular polymeric substances, alteration of adhesion, and modulation of biofilm- or QS-related genes. However, these mechanisms can overlap with general antibacterial stress responses; therefore, future studies must distinguish true QS modulation from nonspecific growth inhibition or cytotoxicity (Hu et al., 2024; Afrasiabi et al., 2024).

A major future direction is the use of nanoparticles not only as active antimicrobial agents, but also as delivery platforms for QS inhibitors, quorum-quenching enzymes, phytochemicals, antibiotics, antimicrobial peptides, or nucleic acid-based therapeutics. This strategy is important because many promising anti-QS compounds have poor aqueous solubility, low stability, limited biofilm penetration, rapid degradation, or insufficient accumulation at the infection site. Nanocarriers may improve local concentration, protect fragile molecules, allow controlled release, and facilitate penetration into biofilm structures (Hu et al., 2024; Qu et al., 2024).

Smart nanomaterials may also be engineered to respond to infection-associated stimuli such as acidic pH, bacterial enzymes, redox imbalance, hypoxia, or biofilm matrix components. In principle, these systems could remain relatively inactive in normal tissues and release the anti-QS or antibiofilm agent preferentially in infected microenvironments. This would reduce off-target effects and limit unnecessary exposure of commensal microbiota. Stimuli-responsive antimicrobial and antiadhesive coatings are therefore gaining attention for medical devices, where bacterial colonization and biofilm formation remain major causes of persistent infection (Cassa et al., 2024).

Despite these advantages, nanoparticle-based anti-QS strategies require careful safety evaluation. Nanoparticles may interfere with colorimetric or fluorescent assays, alter bacterial metabolism nonspecifically, induce oxidative stress in host cells, accumulate in tissues, or produce inflammatory responses depending on their physicochemical properties. Future studies should therefore include cytotoxicity assays, hemocompatibility testing, inflammatory markers, environmental safety assessment, and comparison between QS-specific effects and broader antimicrobial stress responses (Afrasiabi et al., 2024; Hu et al., 2024).

12.3 Combination therapies and synergistic approaches

Anti-QS compounds are unlikely to replace antibiotics in the immediate future, but they may become valuable adjunctive agents. Their most realistic translational potential lies in weakening pathogenic behavior, reducing biofilm protection, decreasing virulence factor production, increasing antibiotic penetration, and improving immune-mediated clearance. This approach is attractive because anti-QS agents do not necessarily need to kill bacteria directly; instead, they may render bacterial populations less aggressive and more susceptible to conventional antimicrobial treatment (Wang et al., 2024; Naga et al., 2024).

Several recent studies and reviews have emphasized the potential of combining quorum-sensing inhibitors with antibiotics to combat bacterial resistance and biofilm-associated tolerance. Such combinations may be useful because QS controls biofilm development, extracellular matrix production, efflux-related responses, and virulence factor expression, all of which may influence antibiotic efficacy. Therefore, a QSI that has limited antibacterial activity on its own may still be clinically valuable if it increases the effectiveness of an antibiotic or reduces the concentration required to control infection (Wang et al., 2024).

Future combination studies should be designed more rigorously than simple co-treatment experiments. A reduction in biofilm biomass after exposure to a QSI–antibiotic combination should not automatically be interpreted as synergy. Synergistic interactions should be assessed using checkerboard assays, fractional inhibitory concentration indices, time-kill kinetics, biofilm eradication assays, Bliss independence models, Loewe additivity, or response-surface approaches. In addition, the effect should be interpreted in relation to bacterial growth, viable biofilm burden, QS gene expression, virulence factor production, and signal molecule levels (Wang et al., 2024; Filipić et al., 2024).

Combination therapy may also involve QS inhibitors with nanoparticles, antimicrobial peptides, quorum-quenching enzymes, matrix-degrading enzymes, or surface coatings. These combinations are promising because biofilm-associated infections are rarely controlled by a single mechanism. One agent may reduce QS-regulated virulence, another may destabilize the extracellular matrix, while an antibiotic may kill metabolically active cells. This multi-target logic is particularly relevant for chronic wounds, cystic fibrosis lung infections, catheter-associated infections, dental biofilms, and implant-related infections (Mitra et al., 2024; Qu et al., 2024).

Another important future direction is temporal combination therapy. QS inhibition may be more effective during early adhesion and biofilm maturation, whereas mature biofilms may require matrix disruption, antibiotic penetration enhancement, or physical removal. Therefore, future studies should distinguish between preventive, early-intervention, mature-biofilm, and recurrence-prevention models. This distinction is essential because the same anti-QS compound may show strong activity against early biofilm formation but limited efficacy against established biofilms (Mitra et al., 2024; Wang et al., 2024).

12.4 Anti-biofilm biomaterials and surface engineering

Surface engineering is a major emerging direction for preventing infections associated with catheters, orthopedic implants, dental materials, contact lenses, wound dressings, and other medical devices. Many device-associated infections begin with bacterial adhesion to abiotic surfaces, followed by microcolony formation, QS activation, extracellular matrix production, and mature biofilm development. Anti-QS strategies can therefore be incorporated into biomaterials to interfere with bacterial communication at the earliest stages of surface colonization (Qu et al., 2024; Priyadarshini et al., 2024).

Anti-QS biomaterials may include immobilized quorum-sensing inhibitors, quorum-quenching enzymes, nanoparticles, carbon dots, antimicrobial peptides, hydrogels, polymeric coatings, or stimuli-responsive release systems. Their main advantage is local action at the site where biofilm formation begins. This local effect may reduce systemic exposure, maintain effective concentrations near the bacterial community, and limit early QS-mediated coordination. Materials with anti-QS properties are therefore increasingly studied as tools for preventing biofilm formation rather than only treating established infections (Qu et al., 2024).

Carbon dots and other nanostructured materials are attracting particular attention for medical device and implant applications because they may combine antibiofilm activity, surface functionalization capacity, biocompatibility, and tunable physicochemical properties. In this context, carbon dot-based coatings are being investigated as potential strategies to reduce bacterial attachment and biofilm formation on medical devices and implants (Priyadarshini et al., 2024).

Stimuli-responsive coatings represent another important direction. These systems are designed to remain stable under normal conditions but release antimicrobial or antibiofilm agents in response to bacterial colonization or infection-associated signals. Bacteria-triggered coatings are particularly attractive because they may reduce unnecessary drug release while providing a localized defensive response when colonization begins (Cassa et al., 2024).

However, anti-biofilm biomaterials face several methodological and translational challenges. The coating must remain stable under physiological conditions, retain activity over time, avoid cytotoxicity, preserve host-cell compatibility, and maintain the mechanical and functional properties of the device. Moreover, static in vitro adhesion assays are insufficient to predict clinical performance. Future biomaterial studies should include flow conditions, protein conditioning, polymicrobial communities, immune components, long-term stability testing, and ex vivo or in vivo device-associated infection models (Cassa et al., 2024; Qu et al., 2024; Priyadarshini et al., 2024).

The most convincing future studies will not merely show reduced crystal violet staining on a coated surface. They should demonstrate decreased viable biofilm burden, altered QS-regulated gene expression, reduced virulence factor production, maintained biocompatibility, coating stability, and protection in models that approximate clinical device-associated infection (Qu et al., 2024; Filipić et al., 2024).

12.5 Microbiome engineering and quorum modulation

A major limitation of many antibacterial and antibiofilm strategies is that they target pathogens without sufficiently considering the surrounding microbiome. In real infections, pathogenic bacteria interact with commensal microorganisms, host metabolites, immune mediators, nutrient gradients, and competing microbial communities. QS is deeply involved in these interactions because bacterial signals may influence not only members of the same species but also neighboring species and host responses. Future anti-QS research should therefore move from pathogen-centered inhibition toward ecosystem-level quorum modulation (Naga et al., 2024; Alum et al., 2025).

Microbiome engineering may offer new ways to control pathogenic QS without broadly disrupting beneficial microorganisms. Engineered probiotics, synthetic microbial consortia, quorum-quenching bacteria, phage-based tools, and outer membrane vesicle-based delivery systems could theoretically degrade pathogenic signals, produce competing signal analogues, deliver quorum-quenching enzymes, or reshape microbial community behavior. This concept is supported by the growing development of engineered microbial systems capable of sensing environmental signals and producing therapeutic responses (Guo et al., 2025).

Quorum-quenching enzymes are particularly relevant in this context because they can interfere with QS by degrading or modifying autoinducer molecules rather than killing bacteria directly. Lactonases, acylases, oxidoreductases, and other quorum-quenching enzymes may reduce pathogenic communication and biofilm formation. However, enzyme-based strategies require improved formulation, stability, delivery, and persistence under real-world conditions. Recent work on durable

formulations of quorum-quenching enzymes highlights the importance of enzyme stability and compatibility with applied delivery systems (Jacobson et al., 2025).

Quorum-quenching bacteria also represent a promising biological approach. Certain environmental, endophytic, or commensal bacteria can interfere with QS-regulated phenotypes by degrading signal molecules or producing antagonistic metabolites. Such organisms may inspire future probiotic or microbiome-based strategies for controlling pathogenic communication in specific ecological niches. However, their use requires careful evaluation of safety, colonization capacity, host compatibility, and ecological consequences (Fawzy et al., 2025).

The main challenge in microbiome engineering is predictability. A QS-modulating intervention may have different effects depending on microbial community composition, infection site, immune status, antibiotic exposure, diet, host physiology, and local nutrient conditions. Future studies will therefore need to combine metagenomics, metatranscriptomics, metabolomics, spatial imaging, synthetic ecology, and mathematical modeling to understand how anti-QS interventions affect microbial communities as dynamic ecosystems rather than as isolated bacterial cultures (Guo et al., 2025; Alum et al., 2025).

12.6 Personalized anti-virulence therapies

Personalized anti-virulence therapy is an emerging but highly promising direction. The same anti-QS compound may not work equally well against all strains of a pathogen because QS circuits vary according to species, strain background, mutation profile, virulence repertoire, biofilm-forming capacity, resistance phenotype, and infection environment. In *Pseudomonas aeruginosa*, for example, Las, Rhl, Pqs, and IQS systems interact in a hierarchical and context-dependent manner, and clinical isolates may display altered QS regulation compared with laboratory strains. In *Staphylococcus aureus*, agr-mediated regulation is also strain-dependent and may vary according to infection type, biofilm state, and host conditions (Mitra et al., 2024; Naga et al., 2024).

A personalized anti-QS approach would seek to match the anti-virulence intervention to the regulatory and phenotypic profile of the infecting strain rather than assuming that a single QSI will be universally effective. The effectiveness of anti-QS compounds may depend not only on the presence of a particular signaling pathway but also on its activation level, the stage of infection, biofilm maturity, microbial interactions, and the physiological state of the bacterial population. Consequently, successful implementation of anti-virulence therapy will require a detailed understanding of the biological context in which QS operates.

Such an approach would require rapid diagnostic tools capable of identifying the pathogen, detecting key virulence genes, measuring QS activity, assessing biofilm phenotype, determining antibiotic susceptibility, and possibly characterizing the local microbiome. Molecular diagnostics, biosensor assays, transcriptomics, metabolomics, and AI-supported decision tools could then be combined to guide treatment selection (Almatroudi, 2025; Filipić et al., 2024). Future diagnostic systems may generate comprehensive virulence profiles revealing active communication pathways, expressed virulence determinants, and the anti-QS interventions most likely to produce clinically meaningful effects.

This approach is particularly relevant for chronic and recurrent infections, including cystic fibrosis lung infections, chronic wounds, diabetic foot infections, urinary catheter infections, periodontal biofilms, and implant-associated infections. These infections are often polymicrobial, biofilm-associated, and shaped by patient-specific factors. In such settings, classical MIC-based treatment decisions may be insufficient because the clinical problem is not only bacterial growth but also biofilm persistence, immune evasion, tissue damage, and virulence regulation (Mitra et al., 2024; Wang et al., 2024). Chronic infections frequently evolve over time, with shifts in microbial composition, virulence expression, and host responses, making adaptive therapeutic strategies particularly valuable.

The importance of personalization becomes even more evident in polymicrobial communities. Quorum sensing frequently operates within ecological networks rather than isolated bacterial populations, and interactions among species may profoundly influence virulence expression and treatment outcomes. Consequently, future anti-QS strategies may need to consider not only the target pathogen but also the broader microbial ecosystem in which it resides.

Personalized anti-virulence therapy may involve different strategies depending on the infection context. Some cases may benefit from a QSI–antibiotic combination; others may require nanoparticle-mediated delivery, anti-biofilm biomaterials, quorum-quenching enzymes, phage-based modulation, or microbiome engineering. The choice should be guided by the pathogen's QS architecture, the stage of biofilm development, the infection site, the host immune condition, and the safety

profile of the intervention (Hu et al., 2024; Qu et al., 2024; Jacobson et al., 2025). Nanocarriers, smart biomaterials, and controlled-release platforms may further improve local delivery and therapeutic efficiency.

The integration of artificial intelligence into personalized anti-virulence therapy also represents a potentially transformative development. AI-assisted systems may help identify patterns linking pathogen genotype, virulence phenotype, biofilm behavior, antimicrobial susceptibility, and patient characteristics. Such tools may also accelerate the discovery of new QS inhibitors and facilitate the identification of patient populations most likely to benefit from specific interventions (Almatroudi, 2025; Filipić et al., 2024).

Nevertheless, personalized anti-QS therapy will require clearer translational frameworks. Anti-QS compounds should be evaluated not only for their ability to reduce virulence markers in vitro, but also for their impact on disease severity, tissue damage, immune clearance, recurrence, microbiome stability, and host safety. Regulatory pathways may also need to adapt, because anti-virulence agents do not always fit conventional antimicrobial categories based on bacterial killing or growth inhibition.

The most credible future for anti-QS research will therefore be integrative: AI-assisted discovery to identify candidates, smart nanomaterials to improve delivery, combination therapy to enhance efficacy, biomaterials to prevent device-associated biofilms, microbiome engineering to modulate ecological communication, and personalized diagnostics to select the right intervention for the right infection context (Filipić et al., 2024; Wang et al., 2024; Guo et al., 2025). By combining advances in diagnostics, computational biology, biomaterials science, microbiome research, and anti-virulence pharmacology, personalized anti-QS therapy may provide more precise and biologically informed strategies for managing chronic, biofilm-associated, and multidrug-resistant infections.

Given the methodological complexity of anti-quorum sensing research and the growing diversity of validation approaches, an integrated experimental framework is required to distinguish genuine quorum sensing inhibition from nonspecific antimicrobial, antibiofilm, or metabolic effects. The recommended workflow for validating anti-QS activity is summarized in Figure 8.

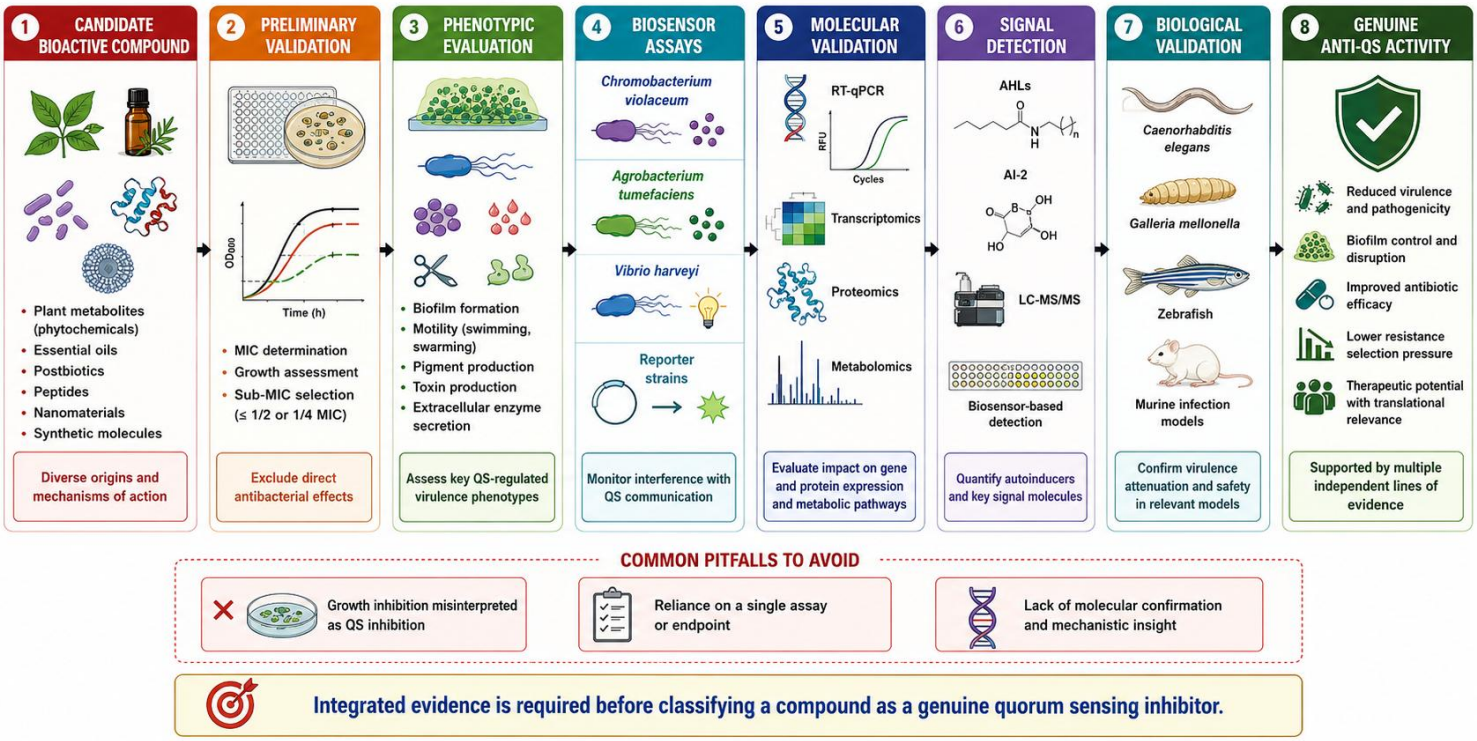

Figure 8. Recommended workflow for validating anti-quorum sensing activity

13. Quick pathogen-oriented guide for anti-QS studies

Anti-QS investigations should be adapted to the biological features of each pathogen because quorum sensing systems, signal molecules, virulence outputs, biofilm behavior, and experimental readouts vary substantially among bacterial species. A pathogen-oriented methodological framework helps researchers select the most relevant QS targets, phenotypic assays,

molecular endpoints, biosensor systems, and validation models. Since the same anti-QS compound may act through receptor antagonism, signal degradation, inhibition of signal synthesis, modulation of virulence-gene expression, biofilm disruption, or indirect metabolic effects, anti-QS claims should always be interpreted within the specific biological context of the pathogen under investigation.

The need for pathogen-specific design becomes evident when comparing major bacterial models. *Pseudomonas aeruginosa* possesses a complex QS network involving Las, Rhl, and PQS systems, whereas *Staphylococcus aureus* relies mainly on the peptide-mediated agr system. Likewise, *Escherichia coli*, *Salmonella enterica*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Streptococcus mutans* exhibit distinct regulatory architectures that influence virulence expression and anti-QS evaluation. Consequently, experimental approaches that are informative in one organism may be unsuitable in another.

Selection of appropriate phenotypic endpoints is critical because QS regulates different virulence determinants depending on the species. In *P. aeruginosa*, pyocyanin production, elastase activity, rhamnolipid synthesis, motility, and biofilm formation are commonly assessed. In *S. aureus*, toxin production, hemolysis, RNAIII expression, and agr-regulated factors are frequently examined. In *E. faecalis*, gelatinase activity and biofilm formation are often relevant, whereas in *S. mutans*, acid tolerance, competence, bacteriocin production, and dental biofilm development may provide more meaningful information. Inappropriate endpoint selection can therefore lead to misleading conclusions.

The nature of the signaling molecules must also be considered. QS inhibition may involve inhibition of signal synthesis, signal degradation, receptor antagonism, disruption of signal transduction, or interference with downstream gene expression. However, the relative importance of these mechanisms differs among pathogens. For example, compounds that degrade acyl-homoserine lactones may be effective against many Gram-negative bacteria but have limited effects on peptide-mediated QS systems in Gram-positive organisms. Similarly, agr-targeting compounds may not influence AI-2-dependent communication pathways. Mechanistic interpretations should therefore remain pathogen-specific.

Molecular validation should likewise be adapted to the QS network being studied. Phenotypic assays alone rarely provide definitive evidence of QS inhibition. In *P. aeruginosa*, genes such as *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, and *pqsR* are commonly targeted, whereas *agrA*, *agrC*, *RNAIII*, *hla*, and *psm* are frequently analyzed in *S. aureus*. In Enterobacteriaceae, *luxS*, *lsr*, *sdiA*, and related regulators may be more informative. Such analyses strengthen the distinction between genuine QS interference and nonspecific effects on bacterial physiology.

The biological model used is equally important. Although planktonic cultures and static biofilm assays are reproducible and widely used, they only partially reflect infection complexity. Mature biofilms, nutrient gradients, host factors, polymicrobial interactions, and immune responses are often absent from conventional assays. Therefore, promising anti-QS compounds should be progressively evaluated using advanced biofilm systems, multispecies communities, organoids, ex vivo tissues, and animal infection models.

Interpretation of biofilm-related results also requires caution. Reduced biofilm biomass does not necessarily indicate QS inhibition because biofilm formation can be influenced by growth impairment, metabolic stress, membrane damage, altered adhesion, or extracellular matrix disruption. Similarly, polymicrobial infections introduce ecological interactions that may modify treatment outcomes in ways not observed in monoculture systems. Negative results should also be interpreted carefully, as inhibition of one QS branch may not affect all virulence-associated phenotypes.

Ultimately, pathogen-oriented methodological frameworks are essential for improving the rigor, reproducibility, and translational relevance of anti-QS research. No single experimental workflow is suitable for all quorum sensing-regulated pathogens. Differences in signaling systems, virulence determinants, biofilm behavior, and host interactions require pathogen-specific methodological considerations. Table 6 therefore provides a practical guide summarizing the most relevant experimental endpoints, molecular targets, validation approaches, biological models, and common interpretation pitfalls for major bacterial pathogens frequently used in anti-quorum sensing research.

Table 6. Quick pathogen-oriented guide for anti-QS studies

Pathogen / bacterial model	Main QS system(s) to consider	Priority phenotypic assays	Recommended molecular / analytical validation	Best experimental use	Recommended in vivo model	Key interpretation cautions	References
Pseudomonas aeruginosa	Las, Rhl, PQS and IQS-associated networks	Biofilm assay, pyocyanin quantification, elastase activity, protease activity, swarming, swimming, twitching motility, rhamnolipid-associated assays	RT-qPCR of lasI/lasR, rhlI/rhlR, pqsA/pqsR; LC-MS/MS for AHLs, PQS/HHQ, phenazines and rhamnolipids; CLSM for biofilm architecture	Reference pathogen for anti-QS, antibiofilm, antivirulence, nanomaterial and combination studies	*Galleria mellonella*, zebrafish larvae, murine lung infection model, 3D lung epithelial model, lung-on-chip infection model	Reduced pyocyanin, elastase or motility may result from growth inhibition, metabolic stress, iron limitation or assay interference rather than direct QS inhibition	Miranda et al., 2022; Zhang et al., 2025; Gad et al., 2024; Rodríguez-Urretavizcaya et al., 2024; Wen et al., 2024
Staphylococcus aureus	Agr peptide-based QS system	Hemolysis assay, toxin production, protease activity, biofilm biomass, biofilm dispersal, adhesion assays	RT-qPCR of agrA, agrC, RNAIII and toxin genes; ELISA or Western blotting for toxins; secretome analysis	Gram-positive model for peptide-mediated QS, toxin regulation and anti-virulence therapy	*Galleria mellonella*, murine skin/wound infection model, murine catheter-associated biofilm model, 3D skin or epithelial infection model	Agr inhibition may increase biofilm retention while reducing toxins; biofilm reduction alone is not sufficient to prove agr inhibition	Yamazaki et al., 2024; Williams, 2025; Podkowik et al., 2024; Beenken et al., 2025
Chromobacterium violaceum	CviI/CviR AHL-mediated system	Violacein inhibition, biofilm assay, protease activity, pigment quantification	Biosensor-based AHL detection; RT-qPCR of QS-related genes where possible; growth and pigment-interference controls	Preliminary screening of anti-QS compounds, plant extracts, nanoparticles and natural products	*Caenorhabditis elegans*, *Galleria mellonella*, simple infection/survival models for preliminary validation	Violacein reduction is a screening endpoint only; it may reflect toxicity, growth inhibition, pigment interference or altered metabolism	McClean et al., 1997; Blosser & Gray, 2000; Warrier et al., 2021; Naga et al., 2024; Hetta et al., 2024
Vibrio harveyi / *Vibrio campbellii*	LuxM/LuxN, LuxS/LuxPQ, CqsA/CqsS systems	Bioluminescence assay, biofilm assay, motility, secretion-associated phenotypes	Luminescence reporter kinetics; AI-2 and AHL-related signal detection; RT-qPCR of QS regulators	Model for multi-signal QS integration and biosensor-based screening	Artemia nauplii, shrimp larvae, zebrafish larvae, aquatic infection models	Luminescence is metabolism-dependent and may be reduced by general physiological stress, oxygen limitation or ATP depletion	Chu et al., 2024; Warrier et al., 2021; Miller & Gilmore, 2020; Ábrahám et al., 2024
Vibrio cholerae	CAI-1, AI-2 and HapR/LuxO-centered QS network	Biofilm formation, motility, colonization-associated assays, virulence-factor-related endpoints	RT-qPCR of hapR, luxO, cqsA, luxS and virulence-associated genes; signal detection where possible	Model for density-dependent regulation of biofilm, dissemination and intestinal infection	Infant mouse intestinal colonization model, zebrafish larvae, intestinal organoid or gut-on-chip model	QS can repress or activate different traits depending on cell density and infection stage; timing is critical	Sajeevan et al., 2025; Chu et al., 2024
Vibrio vulnificus	LuxS/AI-2 and SmcR-associated regulation	Cytotoxicity assay, biofilm formation, motility, metalloprotease activity	RT-qPCR of luxS, smcR and virulence genes; protease assays; host-cell cytotoxicity validation	Useful for foodborne, marine-associated and opportunistic infection studies	Zebrafish larvae, murine septicemia or wound infection model, aquatic host models	Virulence attenuation may reflect reduced growth, stress sensitivity or impaired metabolism rather than specific QS disruption	Chu et al., 2024; Ding et al., 2024
Escherichia coli	LuxS/AI-2-associated signaling and SdiA-mediated AHL sensing	Biofilm assay, motility, adhesion, stress tolerance, host-cell adhesion/invasion assays	RT-qPCR of luxS, lsr genes, sdiA and virulence genes; AI-2-related assays; reporter validation	Model for interspecies signaling, gut colonization and polymicrobial communication	*Galleria mellonella*, *Caenorhabditis elegans*, murine intestinal colonization model, intestinal organoids	E. coli does not produce classical AHLs; SdiA detects exogenous AHLs, so interpretation must distinguish	Chu et al., 2024; Juszczuk-Kubiak, 2024; Cui & Kim, 2024

						endogenous from interspecies signaling	
Enterohemorrhagic *E. coli*	AI-3/catecholamine-related QseBC/QseEF systems and AI-2-associated pathways	Motility, adhesion, type III secretion-related assays, Shiga toxin-associated endpoints, biofilm assay	RT-qPCR of qseB/qseC, qseE/qseF, luxS, stx and LEE-associated genes; toxin quantification	Model for host–pathogen chemical communication and anti-virulence studies	Infant rabbit model, murine colonization model, intestinal organoids, gut-on-chip model	Host catecholamine sensing complicates interpretation; anti-QS effects may overlap with stress-response and virulence-regulatory pathways	Chu et al., 2024; Juszczuk-Kubiak, 2024
Salmonella enterica	LuxS/AI-2 and SdiA-mediated AHL sensing	Biofilm formation, motility, adhesion, invasion, stress-resistance assays	RT-qPCR of luxS, sdiA, invasion and biofilm-associated genes; AI-2-associated assays	Foodborne pathogen model for interspecies signaling, persistence and virulence attenuation	*Galleria mellonella*, *Caenorhabditis elegans*, zebrafish larvae, murine oral infection model, intestinal organoids	SdiA-dependent responses require exogenous AHLs; anti-QS interpretation should include invasion and growth controls	Chu et al., 2024; Ding et al., 2024; Cui & Kim, 2024
Klebsiella pneumoniae	AI-2/LuxS-associated QS-like regulation	Biofilm assay, capsule-associated assays, adhesion, fimbrial activity, siderophore production	RT-qPCR of luxS, capsule, fimbrial, siderophore and biofilm-related genes; CLSM; LC-MS for siderophore-related metabolites	Clinically relevant model for MDR, device-associated biofilms and capsule-associated virulence	*Galleria mellonella*, murine pneumonia model, murine urinary tract infection model, catheter-associated biofilm model	Capsule and biofilm changes may be influenced by metabolic state, growth conditions and antimicrobial stress	Juszczuk-Kubiak, 2024; Cui & Kim, 2024; Zhang et al., 2025
Acinetobacter baumannii	AbaI/AbaR AHL-mediated system	Biofilm formation, surface motility, adhesion, persistence, antibiotic-tolerance assays	RT-qPCR of abaI/abaR and biofilm-associated genes; AHL detection; microscopy of biofilms	Important MDR hospital pathogen for biofilm and surface persistence studies	*Galleria mellonella*, zebrafish larvae, murine pneumonia model, murine wound infection model	Reduced biofilm may reflect impaired growth, surface effects or compound-mediated membrane damage rather than QS inhibition	Sun et al., 2021; Pumirat et al., 2024
Burkholderia cepacia complex	CepIR, CciIR and related LuxIR-type systems	Biofilm assay, motility, protease activity, siderophore production, chronic-infection-associated traits	RT-qPCR of cepI/cepR, cciI/cciR and virulence genes; AHL quantification	Respiratory and cystic fibrosis-associated model for chronic infection and polymicrobial communication	*Galleria mellonella*, zebrafish larvae, murine respiratory infection model, airway epithelial models	Species-level differences are important within the complex; strain identification and QS-system confirmation are required	Chu et al., 2024; Warrier et al., 2021; Cui & Kim, 2024
Serratia marcescens	SwrIR and SmaIR AHL systems	Swarming motility, prodigiosin production, protease activity, biofilm formation	RT-qPCR of QS regulators; pigment quantification; AHL biosensor assays	Useful model for pigment-, motility- and enzyme-based anti-QS screening	*Caenorhabditis elegans*, *Galleria mellonella*, zebrafish larvae	Prodigiosin and swarming are strongly affected by medium composition, temperature and surface humidity	Chu et al., 2024; Warrier et al., 2021
Aeromonas hydrophila	AhyIR and LuxS/AI-2-associated systems	Biofilm, motility, hemolysin, protease, lipase and enterotoxin-associated assays	RT-qPCR of ahyI/ahyR, luxS and toxin genes; enzyme activity assays; cytotoxicity assays	Relevant for aquatic, foodborne and opportunistic infections	Zebrafish larvae, fish infection models, *Galleria mellonella*, aquatic host models	Enzyme inhibition may be direct or indirect; enzyme-only and growth controls are needed	Chu et al., 2024; Ding et al., 2024; Warrier et al., 2021
Enterococcus faecalis	Fsr peptide-mediated QS system	Gelatinase activity, serine protease activity, biofilm formation, adhesion, aggregation	RT-qPCR of fsrA/fsrB/fsrC, gelE and sprE; gelatinase assay; biofilm microscopy	Gram-positive model for enzyme-mediated virulence, endodontic infection and device-associated biofilms	*Galleria mellonella*, *Caenorhabditis elegans*, murine catheter-associated infection	Gelatinase reduction should be separated from growth inhibition and direct enzyme inhibition	Yang et al., 2024; Williams, 2025

					model, dental/root-canal ex vivo model		
Streptococcus mutans	ComCDE, ComRS and LuxS/AI-2-associated systems	Dental biofilm assay, acid tolerance, bacteriocin activity, competence-related assays, adhesion to oral surfaces	RT-qPCR of comCDE, comRS, luxS, glucosyltransferase and acid-tolerance genes; multispecies oral biofilm models	Oral pathogen model for dental caries, biofilm ecology and stomatology-oriented anti-QS studies	Rodent dental caries model, ex vivo tooth/enamel biofilm model, saliva-derived multispecies oral biofilm model	pH, sucrose, salivary components and multispecies interactions strongly affect QS and biofilm behavior	Gao et al., 2024; Cui & Kim, 2024
Streptococcus pneumoniae	ComCDE competence system and LuxS/AI-2-associated signaling	Competence assay, biofilm formation, fratricide-associated assays, colonization-related endpoints	RT-qPCR of comCDE, luxS and competence genes; biofilm and host-cell assays	Model for competence-associated virulence, genetic exchange and colonization	Murine nasopharyngeal colonization model, murine pneumonia model, zebrafish larvae	Competence regulation is time-sensitive and condition-dependent; biofilm changes alone are insufficient	Williams, 2025; Juszczuk-Kubiak, 2024
Clostridioides difficile	Agr-like peptide QS and LuxS/AI-2-associated systems	Toxin production, sporulation-associated assays, biofilm formation, colonization-associated endpoints	RT-qPCR of toxin genes, agr-like genes and luxS; toxin ELISA; anaerobic biofilm models	Relevant for intestinal infection, toxin regulation and microbiome-linked anti-virulence studies	Murine antibiotic-associated colitis model, hamster infection model, intestinal organoids, gut microbiota-associated models	Anaerobic conditions, growth phase and toxin regulation must be rigorously standardized	Williams, 2025; Juszczuk-Kubiak, 2024; Ding et al., 2024
Listeria monocytogenes	Agr-like QS and LuxS/AI-2-associated signaling	Biofilm formation, adhesion, invasion, stress tolerance, food-surface persistence assays	RT-qPCR of agr-like, luxS, invasion and stress-response genes; host-cell adhesion/invasion models	Food safety model for surface persistence, stress adaptation and anti-adhesion strategies	*Galleria mellonella*, zebrafish larvae, murine oral or systemic listeriosis model, intestinal organoids	Food-processing conditions and surface type strongly affect biofilm and adhesion results	Ding et al., 2024; Juszczuk-Kubiak, 2024
Helicobacter pylori	LuxS/AI-2-related signaling	Motility, biofilm formation, adhesion, colonization-associated assays	RT-qPCR of luxS, motility and colonization-associated genes; AI-2-related assays	Gastric colonization model for QS-associated persistence and biofilm-like behavior	Mongolian gerbil gastric infection model, murine gastric colonization model, gastric organoids	LuxS is also linked to metabolism; QS-specific interpretation requires careful controls	Juszczuk-Kubiak, 2024; Warrier et al., 2021

14. Conclusion

Quorum sensing has become one of the most strategically relevant targets in anti-virulence research because it occupies a central position between bacterial communication, collective pathogenic behavior, biofilm development, host adaptation, and antimicrobial tolerance. By regulating key virulence-associated traits such as adhesion, motility, toxin secretion, extracellular enzyme production, pigment synthesis, immune evasion, stress tolerance, and biofilm maturation, QS allows pathogenic bacteria to coordinate infection as a population-level process rather than as an isolated cellular event. Targeting this regulatory network therefore offers a conceptually powerful alternative to conventional antimicrobial strategies: instead of directly killing bacteria or suppressing their growth, anti-QS approaches aim to disorganize virulence, weaken pathogenic cooperation, and increase bacterial vulnerability to host defenses and antimicrobial interventions.

The increasing number of studies reporting anti-QS activities of bioactive compounds confirms the scientific vitality of this field. Plant-derived metabolites, essential oils, marine natural products, probiotics, postbiotics, biosurfactants, bacteriocins, antimicrobial peptides, quorum-quenching enzymes, synthetic inhibitors, and nanostructured materials represent a broad reservoir of potential anti-virulence agents. Yet, the same diversity that makes this field promising also makes it methodologically fragile. Many bioactive compounds are chemically complex, biologically pleiotropic, and capable of affecting bacterial physiology through mechanisms unrelated to quorum sensing, including growth delay, metabolic inhibition, oxidative stress, membrane perturbation, pH modification, nutrient depletion, enzyme inhibition, biofilm matrix destabilization, or direct interference with colorimetric, fluorometric, and reporter-based assays. Consequently, reduced biofilm biomass, decreased pigment production, impaired motility, lower toxin secretion, or diminished extracellular enzyme activity should not be interpreted as definitive evidence of QS inhibition in the absence of rigorous controls and mechanistic validation.

A major conclusion emerging from this review is that anti-QS research must move beyond descriptive phenotypic screening toward integrated mechanistic demonstration. Reliable identification of a QS inhibitor requires a stepwise experimental framework that begins with precise MIC determination, confirmation of sub-inhibitory and non-growth-suppressive concentrations, and careful monitoring of bacterial growth kinetics and viability. Phenotypic assays should then be interpreted only in combination with appropriate negative, positive, vehicle, medium, solvent, and compound-specific controls. For complex preparations such as plant extracts, essential oils, postbiotics, and nanoparticles, chemical or physicochemical characterization is indispensable before biological conclusions can be drawn. Likewise, molecular confirmation through RT-qPCR, transcriptomics, proteomics, metabolomics, reporter systems, direct signal molecule quantification, and advanced biofilm imaging is essential to distinguish genuine disruption of QS circuits from nonspecific attenuation of virulence-associated outputs.

The biological model used is equally decisive. Although reference strains remain valuable for reproducibility and comparative screening, they cannot fully capture the heterogeneity of clinical infections. Clinical isolates, multidrug-resistant strains, polymicrobial communities, mature biofilms, ex vivo systems, and in vivo infection models are needed to determine whether anti-QS effects observed under simplified laboratory conditions retain biological relevance in more complex contexts. This is particularly important because QS networks are highly dependent on strain background, growth phase, nutrient environment, oxygen availability, stress conditions, infection site, and interspecies interactions. A compound that appears active in a planktonic monoculture assay may lose activity, acquire a different biological meaning, or produce unexpected ecological effects in multispecies biofilms or host-associated environments.

The future credibility of anti-QS research will depend on standardization, reproducibility, and translational realism. Studies should clearly report bacterial strains, culture conditions, inoculum preparation, compound characterization, concentration selection, assay endpoints, replication strategy, statistical treatment, and criteria used to define QS inhibition. The field also requires stronger harmonization between phenotypic assays and molecular evidence, as well as more systematic use of dose-response analysis, time-course experiments, omics-based profiling, signal detection, cytotoxicity assessment, and infection-relevant validation. Emerging technologies such as artificial intelligence-assisted screening, molecular docking, molecular dynamics, high-content imaging, smart nanocarriers, engineered quorum-quenching enzymes, organoid models, microbiome-based systems, and multi-omics integration will be valuable only if embedded within rigorous experimental designs.

In conclusion, bioactive compounds targeting quorum sensing offer a promising route toward next-generation anti-virulence strategies, particularly for biofilm-associated, chronic, polymicrobial, and multidrug-resistant bacterial infections. However, their scientific and therapeutic value cannot rest on phenotypic inhibition alone. The field must adopt a stricter evidentiary

standard in which anti-QS claims are supported by growth-independent effects, mechanistic specificity, signal-level or gene-level validation, chemical characterization, reproducible controls, and biologically relevant models. Only through this methodological maturation can anti-QS research progress from promising laboratory observations to credible translational applications capable of complementing or reshaping future antimicrobial therapy.

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